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13. ABSTRACT *(Maximum 200 words)*

My laboratory has generated a human mammary epithelial cell (HMEC) system for studies on normal and aberrant growth control mechanisms. As part of the goal to facilitate widespread use of HMEC, we have provided cells and cell culture expertise to over 150 laboratories worldwide.

To optimize use of this resource, we developed a complex database for storage and retrieval of information on the HMEC, and newsletters for communication among those using these HMEC. The purpose of this grant is to (1) upgrade and expand the features of this database, (2) ensure that all computer and written records can be readily understandable to others besides the PI, (3) create written histories of complicated cell lineages and enter them into the database, (4) develop an Email bulletin board to foster rapid, cooperative communication among laboratories using HMEC.

Most work on the database upgrade was completed in Year 1. Only minor additional changes have been necessary. For year 2, old records were entered into the database and work was begun on compiling cell histories and making written records more accessible. A world wide web has been launched. This work is proceeding as planned, although it is clear that the history compilation will require much time.

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5/13/96

PI - Signature Date

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INTRODUCTION

My laboratory's research program has centered on the development and utilization of human mammary epithelial cell (HMEC) cultures. The overall goal of this work has been to generate human epithelial cell systems for studies on the normal mechanisms controlling proliferation and differentiation in human cells, and on how these normal processes may become altered as a result of immortal and malignant transformation. Included in this goal has been the desire to facilitate widespread use of human epithelial cells for molecular and biochemical studies. Therefore, we have endeavored to develop a system that is relatively easy to use, and can provide large quantities of well-characterized, uniform cell populations. We have already provided cells and cell culture expertise to over 150 laboratories worldwide, and new requests continue to be received.

During the past 18 years, we developed an HMEC bank which contains the following types of material: (1) primary cells (frozen as epithelial organoids or cell clumps) from reduction mammoplasties, tumors, non-tumor mastectomy tissue, benign tumors, and gynecomastias from nearly 200 individuals; (2) higher passage pools of single cells from the above tissue types ; (3) cells from reduction mammoplasty specimens that have been exposed to benzo(a)pyrene and have acquired extended life in culture; (4) the immortally transformed 184A1 and 184B5 cell lines, including clonal isolates, and spontaneous and carcinogen induced variants of these lines; (5) malignantly transformed derivatives of 184A1 and 184B5. In the past year, we have also generated three new immortally transformed cell lines, although these are not yet published and ready for distribution.

The widespread usage of these many HMEC types necessitated development of an appropriate database for information storage and retrieval. A 4th Dimension Database was developed in 1987. One purpose of this grant is to upgrade and expand the features of this database, and to ensure that all computer and written records can be readily understandable to others besides the PI. The second major purpose of this grant is to set up an E-mail network among laboratories using HMEC. Originally, we distributed HMEC newsletters in order to foster rapid, cooperative communication among the groups using HMEC. The new technology of the Internet provides a better avenue for this function.

BODY

With reference to the specific aims of the proposal:

Update computer database

In Year One, as scheduled, we completed most of the work on the 4th Dimension Database upgrade. These changes have been extremely valued, and thus far have worked as intended, so that no further major changes currently appear necessary. Minor corrections and changes have been made as necessary. We will need a programmer in Year Three to make changes in the Search program because of the new lines we are generating. Further work is still required for documentation of use of the Database

Update information in the database

As scheduled, the old records have been added to the database. Those records which had been entered into the inventory file, but not the location file, have had the location information entered. This work is completed.

We have begun checking the actual inventory in the freezers with what exists in computer records. This is difficult tedious work, and will require Year Three for completion.

Update written records on cell cultures

I have begun reviewing old records to (1) rewrite when necessary to make intelligible; (2) distill histories which can be entered into the computer. I started with what I knew were the absolutely worst records (the ones that inspired us to change our record keeping procedures). These were the records of one of our three early experiments [the 184 C (cross) series] in which normal HMEC were exposed to a chemical carcinogen, from which extended life cultures and immortally transformed cell lines were obtained. As expected, this was very difficult. It took the equivalent of around 6-7 full working days, and a small amount of information could not be deciphered. However, in the end I was able to rewrite the records in a

way that others in the lab attested to being comprehensible, and to distill histories which were added to the database (see appendix A, as an example), which other lab members considered sufficient for their needs. I next worked on the 184 B (birdie) series, which was in better condition to start, but is a much more extensive file. This work is taking longer than I had anticipated, and it is clear to me that I will need to continue this for a long time, if these cells are to remain generally useful. I have not done any further old histories in the past year, but have hopefully learned from this experience. In the past year, I have isolated many new clones from the 184 B series immortal cell line (184B5) which have significant biological interest. Additionally, we have generated three new immortal cell lines from the extended life culture 184Aa [of the 184 A (aleph) series]. We are being much more careful to take the time, now, to document the history of these cell cultures as we go.

Another aspect of this work is to go through our 18 notebook files of written records to ensure that records are filed in the appropriate places (many are not). I have not approached this goal systematically in Year Two, and have instead worked on the HMEC records I have needed to use, and reordered them. In some cases, this has required setting up new filing categories that will make it easier for others to file correctly in the future. This work has made clear the large number of incorrect filings that have occurred. There should be no difficulty in completing this by Year Three.

Set up an Email network and communications network

This has been a major thrust of ourwork in Year Two. I have worked mostly with a student assistant to set up the first part of our EMail network. My initial goal was to post on the Internet most of the information that I now routinely need to send to or talk about with other investigators. Therefore, I have set up a web page (see Appendix B; Internet address is http://www.lbl.gov/~mrgs) to let others know what cell types are available, how to obtain cells, an extensive review of the HMEC system (highlighting the most relevant points), and procedures for the use of these cells. Our previous newsletters are also posted. We have posted a list of the other investigators using HMEC, and University of California legal forms, but both these sections still need some work. An announcement has been sent out to those on my newsletter list for whom I have an Email address (see Appendix C). I am also sending by fax or mail an announcement to the rest of the mailing list, along with a request for an Email address.

Our plans for Year Three are to collect and post information from other HMEC investigators, and to make the web site interactive. Additionally, from my observations thus far, I see that it would be beneficial for me to add more information and procedures from my lab (on which I am often queried) to the web site.

CONCLUSIONS

The work has been proceeding largely as planned. The Database has been updated, old records have been entered, the reordering of filed records is proceeding well, and the Email bulletin board has been successfully launched. The two slow aspects of the project are the checking of the freezer inventory, and the compilation of the histories. What I have learned is that it will take me a long time, well beyond Year Three, to do what I think is required for the histories. Therefore, for this granting period I am concentrating on those histories for which there is the most urgent need for review and compilation - namely the experiments that led to our Extended Life cultures and immortally transformed cell lines. I have also learned the importance of keeping good ongoing histories for our newly developing lines.

Requests for HMEC and information about HMEC continue. We have sent cells to around 30 investigators in the past year, and responded to additional queries on HMEC use and biology. Normal HMEC based on our laboratory methods are also being distributed by Clonetics Corp. and the Coriell Institute of NIA, and I am sometimes called with questions from investigators who have received cells from these sources. Therefore, I still feel the need to ensure that our cells, and information about them, can be readily accessible. Already, it is a relief to be able to refer others to our web site for information and procedures. Extending the content of this web site and making it interactive should be a further valuable addition.

APPENDIX A

The following information is placed in the computer database under the "search" category of 184 cross:

184 cross (C)

This was the second in the series of three experiments in which primary cultures of specimen 184 growing in MM were exposed to the chemical carcinogen benzo(a)pyrene (BaP) in the hope of obtaining immortally transformed cell lines. It followed the same general protocol as the first experiment.

Since the first series had been named "aleph" for the first letter of the Hebrew alphabet, I decided to be ecumenical and call the second series "cross".

The experiment was begun on 8/24/82 by seeding 1 ampoule of 184 organoids into 5 T-25 with MM, and 4 T25 with MCDB 202. Flasks G,H in MM received 1µg/ml BaP on days 7 and 10. Flasks E,F received DMSO. Flasks C,D in 202 received 1µg/ml BaP in BSA on day 10. Flasks A,B received DMSO/BSA.

Our record keeping for this experiment was truly horrendous. It was in fact the inspiration for developing uniform cell record keeping sheets and protocols. However, these were not instituted until four months into the experiment. Prior to that time, cells growing in different media were recorded on the same sheet (medium not always indicated) and some naming was arbitrary and repeated. Additionally, many entries are clearly incorrect (i.e., referring to culture passages and dates that didn't exist as recorded). I went over all these records and re-wrote them by current protocols. These are filed along with the original notes under 184 cross.

In summary:

<u>184† in MM</u>
BaP treated cells produced extensive (the most of the three experiments) Extended Life cultures: Ca, Cb, Cc, Cd, Ce, Cf, Cg; but no instances of immortalization. See also histories under individual EL listings (Cb, Cc, Ce, Cf, Cg) for specific information on each EL culture for which there are EL freezedowns. See below for others. See also Figure 3 from EMail review for more information.

<u>ELCa</u>:
This was derived from subculture BP1 of 184† . This was PT1 on day 8 of seeding following 24 hrs after the 1st BaP treatment. Cells were also frozen (AA027).
p2-3: cells were passaged and frozen (AA041)
p3-4: cells were passaged separately, becoming BP1A and BP1B.
p4: most cells aging, a few growing areas in both A and B. Dishes were PTed
p5: some dishes discarded for no growth or contamination, including all BP1A; some BP1B still with growing patches
p6: some growing patches among aging
p7: good uniform growth from p6 patches
p8: some good patchy and uniform growth, cells so-so
p9: no growth. END

<u>ELCd</u>:
This was derived from subculture BP4 of 184†. This was PT7 on day 23 of seeding. PT6 was discarded.
p2: growing and aged cells
p3: good growing areas
p4: several good growing areas per dish. Some dishes PTed
p5: still good growing areas
p6: mostly poor with some OK growth. Dishes discarded
p7: one large swirly patch (picture in PNAS paper, looks just like 184Aa did). Patch grew well after a PT but got contaminated with yeast
p8: (PT of above) discarded, no growth. END

<u>184† in MCDB 202</u>
BaP treated cells: note - I don't think IP was given to these cells, at least through selection, because I was trying to create a more stringent situation. So it could be significant that only the BaP treated 184 cells in 202 gave rise to post-selection cells in the absence of IP.

PT1 following a DT on day 11 of seeding following 24 hrs after the 1st BaP treatment. Subcultures were maintained. BaP treated cells were also frozen (AA034).
On day 15 primary flasks C,D (BaP treated) were both contaminated
p2: good growing
p3: patches of growing cells in many (?all) dishes. Dishes got named A,B,C (which gets confused with C for control and the A,B control flasks; things get majorly difficult to follow)
p4: presumably post-selection cells growing well in what is now called BPA or BP1A, BPC, BP1AB. Freezedowns (records obscure here at points too; BP1A: AA088 great heavy cft ep. in 2 patches; is a freezer record of a BP1C AA101 but this isn't found in the cell records; cell records do have cells frozen at p5 for a BPA which isn't in the freezer records)
p5: good growing. Freezedowns (BP1A: AA113 good cft; BP1C: AA123 great cft)
p6: good growing. Freezedowns (BP1AB: AA125 great cft; BP1C: AA142 great cft; AA140 no record)
p7: good growing
p8: OK growing. Freezedown (BP1A: AA156 OK cft-subcft)
p9: OK so-so, cells getting elongated. Freezedown (BP1C: AA163 OK almost cft)
p10: OK slowly growing
p11: OK so-so yukky. BP1A discarded by here for aging
p12: OK slow growing
p13: OK slow growing. Freezedown (BP1C: AA218 OK cft)
p14: so-so yukky. Aging. Discarded. END
Note: this seems like premature aging for post-selection 184. Perhaps due to the absence of IP during selection. See also later post-selection 184† from AA043 which also had premature aging for 184.

Control cells: note - I don't think IP was given to these cultures, which may explain the failure to yield post-selection cells
PT1 following a DT on day 11 of seeding following 24 hrs after the 1st control treatment. Subcultures were maintained.
PT2 on day 15. Freezedown AA043
PT3 on day 18. Freezedown AA056. Flask A over trypsinized and discarded.
PT4 on day 21. Freezedown AA061. Discard flask B
p2: good growing
p3: mostly feathers, some small growing areas
p4: nothing grew post-selection. END

The following information is placed in the computer database under the "search" categories of ELCb, ELCc, ELce, ELCf; ELCg:

ELCb: see also listing for 184† and Figure 3 from EMail review.
This was derived from subculture BP2 of 184† . This was PT3 on day 11 of seeding following 24 hrs after the 2nd BaP treatment. Cells were also frozen (AA033). PT2 was just frozen (AA029)
p2-3: cells were passaged and frozen (AA050)
p3-4: cells were passaged to 6 60s becoming BP2A-F.
p4: Patchy growth; dishes were PTed
p5: Patchy growth to confluence. Lineages from p4 kept separate. Some dishes discarded for no growth or contamination (not clear).
p6: Mixed good growth on some dishes; cells OK- so-so. Freezedowns (ELCb-E: AA089; good cft mixed small & elongated cells; ELCb-C: AA092; very cft; ELCb-F: AA097; overly cft elongated ep., AA099, cft so-so some elongated cells)
p7: some good growth and dishes discarded with no growth. Freezedowns (ELCb-C: AA091, cft OK so-so; AA100 cft so-so some elongated cells; ELCb-D: AA094 good cft OK; ELCb-A: AA096 overly cft elongated ep; ?ELCb-B: AA103 almost cft so-so)
p8: some dishes still with good growth, most discarded. Freezedowns (ELCb-D: AA118 almost cft good cells, a lot of small cells; AA141 OK cft-subcft)
p9: only ELCb-D maintained, still some growth
p10: no growth or contaminated. END

Some cells were also switched to MCDB 202 at various passages. They were also eventually discarded, with no freezedowns.

An ampoule of AA033 was also started (I don't know why) and it was called BPA (I don't know why, particularly since this name was also used for the 184† cells grown in 202). Technically, this is also BP2. It had mixed (good and aging) growth at p3-4 , reached confluence at p5 (cells so-so), was discarded at p6 (?contamination or no growth).

ELCc: see also listing for 184† and Figure 3 from EMail review.
This was derived from subculture BP3 of 184† apparently flask G only. This was PT5 on day 17 of seeding. PT4 was just frozen (AA035)
p2: cells OK so-so
p3: mixed growing and aging. Cells were passaged separately, becoming BP3A-D
p4: some good growing patches; dishes were PTed
p5: mixed, patches, aged, uniform growth
p6: some patches maintained growth, some dishes discarded. Freezedown (ELCc-C: AA136 almost cft,, a lot of small cells, good)
p7: some patches maintained growth, some dishes discarded. Freezedown (ELCc-A: AA117 almost cft, good, a lot of small cell patches between older cells which form striations; ELCc-C AA138 cft, so-so)
p8: some good growing patches, some dishes discarded
p9: some dishes still had OK-good growth, some discarded. ELCc-C seems the one that maintained growth best (could be just didn't get contaminated)
p10: discarded (no growth or contaminated). END

Some cells were also switched to MCDB 202 at various passages. They were also eventually discarded, with no freezedowns.

ELCe: see also listing for 184† and Figure 3 from EMail review.
This was derived from subculture BP5 of 184† flask G only. This was PT8 on day 29 of seeding.
p2: aging and growing cells
p3: aging and good growing
p4: widespread good growth
p5: reached confluence, cells OK-good
p6: reached confluence
p7: still good growing cells. Freezedown (AA110 very cft so-so)
p8: still good growing cells. Freezedown (AA116 good small cft)
p9: mixed growth to confluence. Freezedown (AA130Z very cft good small cells)
p10: discarded. END

Some cells were also switched to MCDB 202 at various passages. They were also eventually discarded, with no freezedowns.

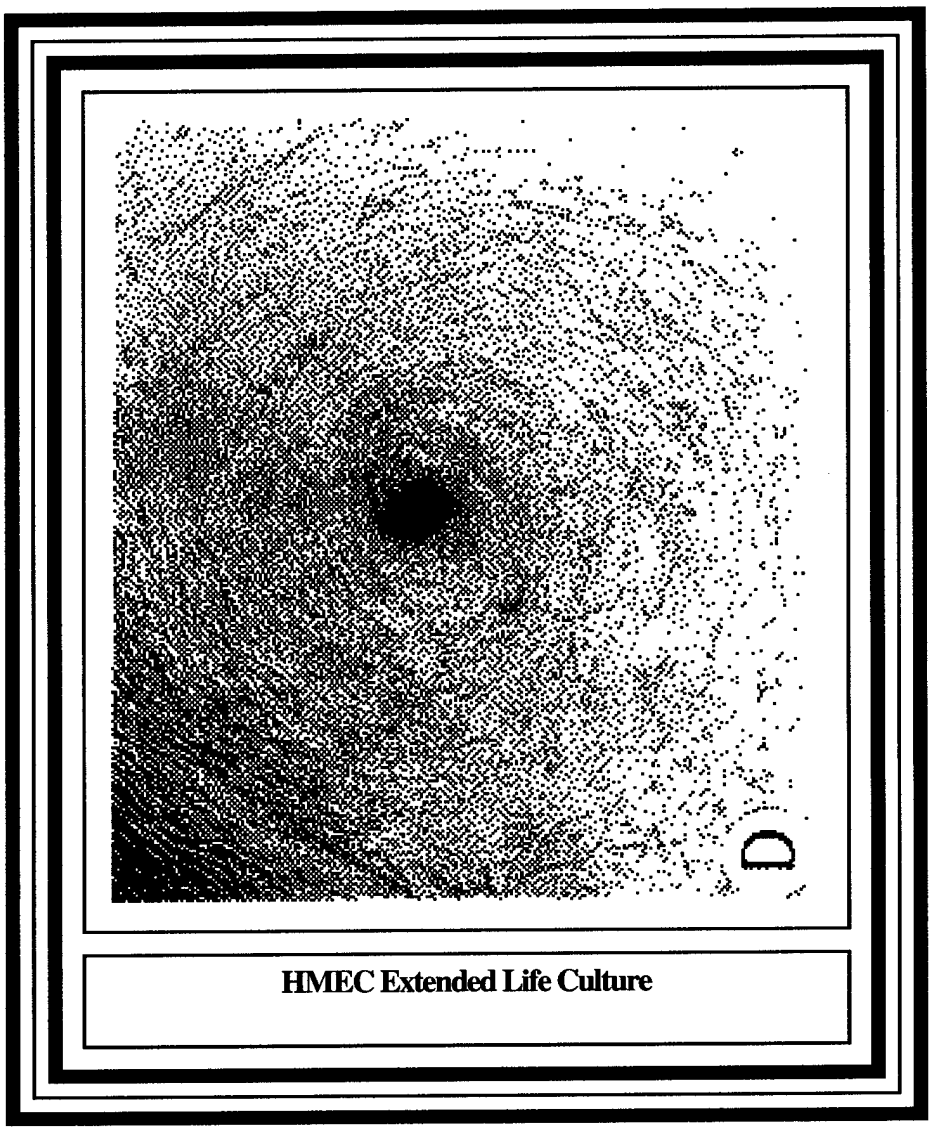
ELCf: see also listing for 184† and Figure 3 from EMail review.
This was derived from subculture BP6 of 184† flask H only. This was PT8 on day 40 of seeding. Primary flask had one large good growing clonal area and three other growing areas
p2: good growing cells
p3: so-so cells. Dishes discarded, not indicated if no growth or contaminated.
p4: some good growth. Dishes discarded, not indicated if no growth or contaminated.
p5: still good growing areas. Some dished PTed. Freezedown (AA150 subcft good-OK)
p6: good growing areas
p7: still good growing patches
looks like massive contamination loss around this time
p6-8: survivors discarded eventually for no growth. END

ELCg: see also listing for 184† and Figure 3 from EMail review.
This was derived from subculture BP7 of 184† flask G only. This was PT10 on day 41 of seeding. Primary flask had several good growing areas. PT 9 was discarded
p2: good growing cells
p3: good growing cells. Freezedown (AA090 good cft)
p4: reached confluence, good growing areas. Freezedown (AA106, AA107 cft good)
p5: aging and growing cells. Freezedown (AA120 subcft-cft some heavy growing areas, a lot of small cells)
p6: so-so cell

p7: lost to contamination. Started again with freezedown AA120 (on same sheet). Following are from this freezedown.
p5: growing and aging
p6: good growing and aging
p7: reached confluence, cells OK
p8: mostly aging, some growing. Freezedown (AA238 so-so cft)
p9: still some growth, but had seeded with "UV" irradiated fibroblasts (one of various schemes I changed in mid-experiment - I haven't mentioned most of these as they don't seem significant) which weren't all so dead and overgrew the cultures. All discarded from fibroblast overgrowth, contamination, or no growth. END

Cells were also transferred to MCDB 202 at various passages. There were some freezedowns.
Transfer to 202 at p4, good cft.
p5: so-so OK good cft
p6: lost to contamination. Freezedowns (AA177 so-so cft; AA191 good cft)

HMEC: Human Mammary Epithelial Cell



HMEC Extended Life Culture

Welcome

This bulletin board provides information on human mammary cell resources that have been developed at my laboratory at the Lawrence Berkeley National Laboratory, University of California. These cells are available for distribution. Information is provided on the cell types available, the derivation of these cells, methods for appropriate use of the cells, and some results which have been obtained in this system. Additionally, the bulletin board is in the process of being developed to allow for interested investigators to post information on their current or prospective research, and to query others for information. A listing is provided of other researchers working on this or related human mammary systems.

As part of my ongoing research program since 1976, my laboratory has developed and stored frozen a variety of normal, benign, tumor-derived, and in vitro transformed cultures of human mammary epithelial cells (HMEC). In the interest of facilitating scientific investigations using HMEC, our laboratory makes these cells available to other researchers. We are not a commercial or official cell bank, and we receive only minor financial support for this service. Please keep in mind the limits of what we c an do. Details describing the cell types available can be found in the sections referred to under "REVIEW OF HMEC CULTURE SYSTEM".

I prefer talking with individual investigators interested in using HMEC. This gives both of us a better understanding of what is most appropriate for the scientific questions being asked. If you want cells, you will be asked to send a brief (1 page) letter describing your planned experiments, and indicating that (1) you will

keep me informed of results or major changes in planned experiments; (2) you will not give the cells to others without my permission. There are also legal forms from the Universi ty of California for you and your institution to sign and return (see "" to download - not in place yet). I will want a FedEx number or equivalent to charge the costs of shipping the cells. I can be reached as follows:

Martha Stampfer
Lawrence Berkeley National Laboratory
1 Cyclotron Road, Bldg. 934
Berkeley CA 94720

Phone-office: 510 486-7273
Phone-lab: 510 486-4558
FAX: 510 486-5735
Email: mrgs@mh1.lbl.gov

Human Mammary Cell Types Available For Distribution
Review Of Human Mammary Epithelial Cell (HMEC) Culture System
Procedures
Postings (not in place yet)(under construction)
Investigator List
Legal Forms [under construction]
Previous Newsletters

If you have trouble printing using netscape you may download text files, using netscape select **save as** from the **file** menu, then select **text** as the format. The current version of netscape can be found here.

Human Mammary Cell Types Available For Distribution

Cells are sent frozen (dry ice) in ampoules containing 5 x 10-5 or 1 x 10-6 cells. Under special circumstances, we can arrange to send growing cells in flasks. (see "REVIEW section VI." for more information on cell shipments)

Finite Lifespan HMEC

(also see section I.)

1. Reduction mammoplasty derived HMEC grown in serum free (MCDB 170 like) medium
Large quantities of post-selection cells are available around passages 7-10 that senesce around passages 15-25 (about 3 population doublings per passage). These cells are routinely available from women of different ages. I most commonly distribute cells from specimens 184, 48R, and 161 (see section I.C. figure 1).

2. Reduction mammoplasty derived HMEC grown in a serum containing (MM like) medium
Limited quantities of cells are available from several individuals. Cells are supplied at 2nd or 3rd passage; most growth ceases around passages 4-5. They contain a greater range of phenotypes than the post-selection MCDB 170 grown cells. I most commonly distribute cells from specimens 184, 48, and 161.

3. Cells from non reduction mammoplasty tissues (i.e., mastectomies; benign tumors)
Limited quantities are available. Cells grown from tumor tissues in MCDB 170 are not reflective of most tumor cells. Talk with me directly about these cells.

Immortally transformed cell lines (section II. & IV.)

1. **184A1** This cell line of indefinite lifespan is available for distribution. For some purposes, it may be preferable to have early or later passage cells, or cells which have been selected for resistance to growth inhibition by TGFb. Clonal isolates are also available.
More limited quantities of cells are available which have been selected for loss of specific nutritional requirements.
Very limited quantities are available of cells which have been infected with oncogenes (H-ras/SV40-T/v-mos).

2. 184B5 This cell line of indefinite lifespan is available for distribution. For some purposes, it may be preferable to have early or later passage cells, or cells which have been tested to be resistant to growth inhibition by TGFb. Clonal isolates are also available.
More limited quantities of cells are available wh ich have been selected for loss of specific nutritional requirements.
More limited quantities are available of cells which have been transfected (erbB-2) or infected with oncogenes (K-ras).

Carcinogen exposed Extended Life cultures (section II.A. a nd figure 3)

Very limited quantities of benzo(a)pyrene treated specimen 184 extended life cultures, including the extended life precursors of the lines 184A1 and 184B5, are available. Talk with me directly about these cells.

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REVIEW OF HMEC CULTURE SYSTEM

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Introduction

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The following reviews the origins and characterization of the HMEC system developed in my lab and those of co-workers. It incorporates most of the information from my lab that was in my four Newsletters. This information will be periodically updated. It probably includes more than you'll ever want to know, but hopefully, someone will find each tidbit valuable and consequently not need to query me on that subject. I welcome feedback on how this information can be presented most usefully.

To put this information in context, my long-term goal (since 1976!) has been to develop an HMEC system that could be used to study the normal mechanisms controlling proliferation and differentiation in human epithelial cells, and to understand how these normal processes become altered as a result of immortal and malignant transformation. Guiding this work was the desire to facilitate widespread use of human epithelial cells for molecular and cellular biology studies, i.e., the hope was that HMEC would seem a reasonable alternative to fibroblasts, or tumor cell lines, or non-human cells. Therefore, I tried to develop a system that is relatively easy to use, can provide large quantities of uniform cell populations, and is relatively well-defined. I realize that "relatively easy" can be in the eyes of the beholder, and for some people HMEC will still seem difficult relative to HeLa or 3T3. While HMEC may require a little more effort, really, they are very easy to grow once you get the hang of it. What is needed is careful attention to proper tissue culture procedures, a basic understanding of the cell system, and a "feel for the organisms". Cells are living creatures, with some resemblance to children - they can behave as if they have minds of their own. As Dick Ham has often said, sometimes what is most important is just to "listen to your cells". The rewards are being able to use cells much closer to relevant human processes, including such things as being able to study growth control in cells with normal human growth control mechanisms.

In developing and promoting the use of the HMEC system, I have been influenced by the following assumptions: (1) Prior knowledge of what constitutes normal cell behavior is necessary to determine what

constitutes abnormal and deranged processes, e.g., if you want to say that something you are studying is a property of a transformed cell, you need also to look at the normal cells. (2) Understanding normal and aberrant human epithelial cell growth control and differentiation will ultimately require examination of human epithelial cells. Non-human and non-epithelial cell studies may provide valuable information and suggest areas of research. However, the many differences which are known to exist between these cell types in culture as well as in whole body physiology indicate that only examination of the cells in question will give an accurate description of those cells' behavior. (3) In a situation where whole animal experiments are not possible (i.e., with human cells), the next best option is to develop culture systems that can as accurately as possible approximate the in vivo state. I have tried to balance the goal of making the system as amenable as possible to widespread use, with the goal of trying to optimizing the system to reflect in vivo biology. The result is considerably less than ideal in terms of in vivo approximation. Normal and aberrant cellular processes in vivo involve complex interactions of polarized cells within three dimension organ systems. Single cell types growing on plastic are not that! Consequently, it's important to remember the limitations of this culture system. I believe it is valuable that work continue in developing culture systems that more accurately mimic in vivo cell-cell and cell matrix interactions.

Since fostering widespread usage of HMEC has been one of my long-term goals, I have tried to make cells available to other interested investigators. I have found it helpful to talk to people individually to understand more precisely their scientific needs and goals in using HMEC. Checking through the relevant parts of this EMail information first can provide a sense of what is available and known. While distributing cells is part of what I enjoy doing, please keep in mind that this is a non-commercial, non-official, personally-run cell bank, and I and my technician are also doing many other things. I offer the HMEC we've developed, and information on their usage. I'm not so open to generic queries about cells, reagents, techniques etc.

Nomenclature notes:

"primary" refers to cells the first time they are placed in culture (e.g., outgrowths from organoids). Cells which have been subcultured are no longer primaries and should not be described as primary culture. I refer to higher passage cultures of normal finite lifespan HMECs as **strains** with **long-term growth potential** in culture. In technical tissue culture parlance, they could be called cell lines once subcultured, but I find this usage confusing and only use **"cell line"** to refer to cells with indefinite growth potential (i.e., immortal). **"extended life"** refers to cells which grow longer than normal as a result of some abnormal in vitro exposure; in my case chemical carcinogens, although it could also be oncogenes. "extended life" should not be used to refer to the HMEC strains with long-term growth since this growth is normal.

I. Derivation of HMEC Cultures

I. A. Tissue Procurement

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We have obtained our human mammary cells from a variety of sources, mostly surgical discard material. What we refer to as normal HMEC is derived from reduction mammoplasty tissues. Women undergoing reduction mammoplasty operations do not have any known epithelial pathology per se (their breasts contain the same amount of epithelial cells as is present in smaller breasts, but they have much more adipose tissue). Their breast tissues do show the range of pathologies generally found in women of the same age (e.g., it may be described as containing mild to atypical hyperplasia, or fibrocystic disease). There is always the possibility that women with such large fat deposits in their breasts could have some abnormality in some aspect of their metabolism. Because large portions of the breast are removed, with minimal need for pathology evaluation, considerable quantities of cells from the same individual are made available from each reduction mammoplasty.

The other major source of tissues comes from mastectomies. Usually the amount of tumor tissue available for culture is small, due to the need for clinical evaluation of the tumor. Larger amounts of the non-tumor peripheral tissue is available. This can be particularly useful in providing matched pairs of tumor and non-tumor tissue from the same person. However, I do not consider peripheral mastectomy tissue as normal, as there is always the possibility of tumor field effects, microtumors within this tissue, field effects from some environmental conditions predisposing to tumors, and inherent genetic abnormalities. For some of the same reasons, I would not view as normal the tissues we have obtained from contralateral mastectomy - tissues removed from the breast contralateral to a tumor-bearing breast for prophylactic or cosmetic purposes.

Additional surgical tissues are obtained from benign conditions: fibroadenomas (which are not thought to be

pre-malignant); fibrocystic tissues (which under some conditions could indicate an increased likelihood of tumor development); gynecomastias, which are benign hyperplasias in male breast tissue.

We also have a few samples of tissues from other conditions. We have two subcutaneous mastectomy tissues. These operations are generally performed because of extensive fibrocystic disease, and in the two samples I processed, the consistency of the tissue appeared grossly abnormal (hard and fibrous) compared to reduction mammoplasties. We have two non-tumor peripheral tissues from breasts that had sarcomas.

Another, non-surgical source of HMEC is from breast fluids. A small number of cells can be obtained from nipple secretions of around 50% of women, and larger volumes are available from lactational fluids. Our original publication in 1980 actually utilized cells from nipple secretions. Cells from milk are valuable as a source of functionally differentiated cells. We have only used these for specific purposes and do not have supplies to distribute.

I. B. Tissue Processing (references: Stampfer et al. 1980; Stampfer 1985 - gives procedure details) Index

Most of the surgically derived tissues are processed by gross selection of epithelial material followed by digestion for 24-72 hrs at 37 °C with collagenase and hyaluronidase. This leaves nearly pure epithelial clumps (termed organoids) which can be separated from the rest of the digested material by collection on filters with pores of fixed size. The organoids can be stored frozen in liquid nitrogen (for at least 18 years - the time since I started this). Material in the filtrate usually contains mainly fibroblastic type cells, and is a good source of matched fibroblasts from the same individual.

The small pieces of tumor tissue are generally not structured in organoids. Digestion for 24 hrs can yield small epithelial clusters and the filtrate may contain many of the single tumor cells. This method is probably not the best available for obtaining tumor cells for culture. It is what was used for the samples that I have stored frozen.

Table 1 gives an idea of what and how many primary tissues we collected and processed.

Table 1: Bank of Primary HMEC Tissue

Tissue Source	# Specimens	Age Range	Median # Ampoules
Reduction Mammoplasty	49	15-66	30
Mastectomy, carcinoma	57	29-93	5
Mastectomy, peripheral non-tumor	43	24-87	8
Mastectomy, contralateral	6	42-77	10
Biopsy (benign tumors)	9	13-47	5
Gynecomastia	6	17-57	9

This represents the amount of tissues as originally collected, rather than current inventory levels. We also have the filtrate material for each specimen, from which, in most cases, fibroblast-like cells can be grown. We are reluctant to give out much primary material, since quantities are limited and we are no longer processing these tissues, but small amounts may be available if essential, particularly for the reduction mammoplasties.

I. C. Media for, and growth of HMEC (references: Stampfer et al. 1980; Stampfer, 1982; Hammond et al. 1984; Stampfer 1985) Index

When I started working with HMEC in 1977, I first developed the MM medium (see **Procedures** for composition of and growth of cells in MM). This medium has a 1:1 DME:F12 base, plus conditioned media from other cell lines, a variety of growth factors, and 0.5% fresh FCS. HMEC obtained from reduction mammoplasties displayed active growth for 2-5 passages. The cultures showed a mixed morphology, with larger, flatter non-dividing cells eventually outnumbering the smaller dividing cells with a cobblestone morphology. I have also employed a number of variations on the MM theme, e.g., with and without a cAMP stimulator (cholera toxin), without the conditioned medium (designated MM4), or without particular growth factors.

While MM provided only a limited amount of cells, this was sufficient to perform many types of experiments. It also provided enough cells to begin more systematic studies on optimizing media for growth of HMEC. This work was done by Susan Hammond in Dick Ham's laboratory, the result of which was the development of the serum-free MCDB 170 medium in 1984. This has a base in which the components have been optimized for HMEC growth, plus a variety of serum-free supplements (see **Procedures** for composition of and growth of cells in MCDB 170). The only undefined element is bovine pituitary extract. When organoids are placed in MCDB 170, there is initial active cell division for 2-3 passages of cobblestone appearing cells. These cells gradually change morphology, becoming larger, flatter, striated, with irregular edges and reduced proliferative capacity. As these larger cells cease growth and die, a small (i.e., a 60 mm dish seeded with 1.5 x 10^-5 cells may show 1-10 areas of active growth) number of cells with the cobblestone morphology maintain proliferative capacity and soon dominate the culture. I called this process, whereby only a small fraction of the cells grown in MCDB 170 display long-term growth potential, **self-selection.** The post-selection cells maintain growth for an additional 7-24 passages (approximately 45-100 population doublings in total), depending upon the individual reduction mammoplasty specimen. At senescence, they appear flatter and more vacuolated, while retaining the cobblestone epithelial morphology. Self-selection can also be observed in primary cultures which are subjected to repeated partial trypsinization, a process wherein approximately 50% of the cells are removed and the remaining cells allowed to regrow. After about 10 partial trypsinizations, most of the cells remaining in the dish display the flat, striated, morphology and cease division. However, nearly every organoid patch also gives rise to areas of the growing cobblestone cells, indicating a widespread distribution of the cell type with long-term growth potential. **NOTE:** if you are trying to take cells through the self-selection process, dishes with the large flat cells may sit there for weeks before the smaller cells become obvious . I suspect that this implies that more is happening than the outgrowth of a pre-existing self-selected population, but we have never investigated this phenomena in depth. **NOTE:** partial trypsinization is a way to obtain more good-growing secondary cultures from primary cultures than if the primaries were fully subcultured. For some reason, the cells in the primaries remain much more vigorous for a longer time period. Perhaps this is due to some mixtures in the primary cell population, or some extracellular matrix material - this question has always intrigued me but it has also never been investigated in depth.

Most of the normal HMEC which I make available (as well as the commercially available cells from Clonetics) represent these post-selection cells which display long-term growth in MCDB 170. These cells are particularly useful in molecular and biochemical studies since they provide a virtually unlimited supply of uniform batches of normal human epithelial cells. Thus, experiments can be repeated using cells from both the same frozen batch, as well as from the same individual. These post-selection cells grow rapidly (doubling times of 18-24 hrs) and will grow clonally with 15-50% colony forming efficiency. However, it is important to remember that the cells with long-term growth potential represent a selected subpopulation of the mammary epithelial cells placed in culture (see below). We have grown a limited number of our frozen primary organoids specimens in MCDB 170, generating large pools of frozen cells for use in our laboratory, as well as for distribution to others. We have thus far grown up cells from 12 reduction mammoplasty tissues, 8 mastectomy tissues (6 tumor tissues, 5 non-tumor, 1 contralateral), and 1 gynecomastia. Figure 1 illustrates the long-term growth potential of cells from each of these individuals. **NOTE:** we have not observed a single instance of spontaneous escape from senescence in the HMEC grown under these conditions. In general, cells from the same individual senesced around the same passage, but there were some exceptions. The following explains how and why we kept track of this information.

Since the post-selection cells in our large freeze-downs may be derived from a small number of presumably clonal outgrowths, it was possible that a single patch with some unusual quality could influence a given freeze-down pool. As a consequence, we gradually (and informally) developed a nomenclature to keep track of the origins of a given cell pool. At the first level, we started using symbols to indicate every time we started a new primary organoid ampoule from the same individual. These were easy-to-write symbols with which to label the dish (e.g., heart , infinity oo, birdie "V", spiral "@", etc.). These are now officially registered in our computer records as **FreezeDownSymbol (FDS)**. Subsequently, we realized that it might be important to also keep track of cell populations coming from different pools of post-selection cells. Each selection pool can be thought of as a different substrate "batch", with the possibility that there might be batch differences. So our FDS may be followed by an indication of **"selection" batch** (e.g., heart-triangle, infinity-3, @K, @L, etc.). These are the symbols present in Figure 1. Visually, cells from the same individual, regardless of batch, tend to have the same characteristic appearance, while we do notice interindividual morphologic differences (see Figure 2). **NOTE:** the most common batch of cells from specimen 184 that I distribute is @K, which senesces around passage 22, whereas most other batches from specimen 184 senesce around passage 18.

Click here to see Figure 1.

Legend to Figure 1: Growth capacity of HMEC in MCDB170 medium. I stopped adding information to this graph several years ago, but this gives the general idea and includes the FDS and selection batch of the cells I most frequently distribute. You can use this to see the expected passage where the cells senesce. Primary cultures obtained from reduction mammoplasties (top two rows) and mastectomies (T= tumor tissue, P= non-tumor tissue from tumor-bearing breast, C= contralateral) were initiated and subcultured with about 8-10 fold amplification per passage. Bottom horizontal lines indicate passage level of initiation of frozen ampoules. Top horizontal lines indicate passage level of no net increase in cell numbers (i.e., senescence is viable; some growth does continue as long as cultures are maintained). Internal horizontal lines indicate that cultures were frozen and reinitiated at that passage. Same shading indicate cells derived from the same "selection". Asterisks indicate cells exposed to a cAM P stimulator during selection. For specimen 184 "V", cultures were initiated from the same primary ampoule but taken through selection with three different cAMP stimulators (cholera toxin, isoproterenol, prostaglandin E1). In a few cases, (indicated by a different shading in primary culture), the tumor cultures were grown in MM in primary culture. [PS: the names of the symbols shown, in order of appearance, are: 161- heart, triangle, newmoon, yinyang, infinity; 184- birdie, spiral (not shown are aleph, cross, lollipop, ecology, flower); 48- silver, orange, pink; 172- icecream, lollipop, diamond; 195L- teardrop, pumpkin; 186T- heartbrk, sunrise]

Other information which may be gleaned from these data: (1) There does not appear to be any loss in viability due to multiple freeze-thaws; (2) There does not appear to be any correlation with growth potential in culture and age of specimen donor. It is possible that the differences seen in growth potential may reflect interindividual differences in optimal growth requirements relative to the nutritional formulation of MCDB170.

Click here to see figure 2.

Legend for figure 2 :Morphology of reduction mammoplasty derived HMEC grown in MCDB 170.
Giemsa stained cultures from
(A) 184 p7; (top image)
(B) 172 p13; (middle image)
(C) 161 p9; (bottom image)

I. D. Characterization of Tissue-derived HMEC (references: Taylor-Papadimitriou et al., 1989, Stampfer & Yaswen, 1992) Index

Since a main goal of studying cells in vitro is to gain understanding of in vivo processes, we have considered it extremely important to characterize the HMEC grown in culture with reference to what is known about human mammary cells in the body. Unlike other organ systems, HMEC in culture (with the exception of lactational fluids and very rare surgical specimens) are not obtained from functionally differentiated tissues (i.e., pregnant, lactating, involuting). Consequently, we have not put much effort into examining these cells for features of functional differentiation. The cells we distribute, growing under standard culture conditions, do not express alpha-lactalbumin or beta-casein. We have instead focused on the type of differentiation we termed "maturation", referring to the developmental history of a cell from a proliferative stem cell population to a cell with diminished reproductive capacity to a "terminally differentiated" cell no longer capable of division. We have been particularly interested in this pathway because human breast tumor cells in vivo and tumor derived cell lines almost uniformly express the phenotype of the most mature normal HMEC in vivo.

The mammary gland consists of pseudostratified epithelia, with a basal layer resting upon a basement membrane and an apical layer facing the lumen of the ducts and alveoli. The basal layer of cells does not contact the lumen, whereas the apical layer may contact the basement membrane as well as the lumen. Apical cells display a polarized morphology, with microvilli at the luminal side. The myoepithelial cells, which contain muscle-like myofilaments, and which contract upon appropriate hormonal stimuli to cause expulsion of milk, lie in the basal layer of cells. Based upon examination of keratin expression and other marker antigens, it has been proposed for the rodent mammary gland that a stem cell population capable of differentiating into both myoepithelial cells and the apical glandular epithelial cells, also resides in the basal cell layer. The actual maturation lineage of human mammary epithelial cells in vivo has not been clearly defined. Based on the rodent mammary gland, and other epithelial tissues, it is reasonable to hypothesize that the most proliferative epithelial population in vivo lies in the basal layer, or intermediate between basal and luminal layers. Conversely, the luminal cells presumably have reduced proliferative capacity, with the most mature and least proliferative cells being those shed into the lumen (and recovered in nipple aspirations and milk fluids). **NOTE:** I do not equate basal cells with myoepithelial cells, and do not believe they are equivalent. Unless one can demonstrate the presence of myofilaments, I do not think a cell should be referred to as myoepithelial.

A variety of studies from Joyce Taylor-Papamidritriou's group and others (see references at end) have defined properties which can be used to distinguish basal vs. luminal human breast cells, cells during lactation, and tumor cells. In general, mammary basal cells, similar to basal cells in stratified tissues such as the skin, express keratins 5 and 14. alpha-actin is present and the calmodulin-like protein (CLP) is preferentially found in the basal cell layer. A subpopulation expresses the common mesenchymal intermediate filament, vimentin. Luminal cells express the keratins 8 and 18 found in simple epithelia like the lung; keratin 19 shows variable expression and the 19 positive cells probably represent the most mature population. In culture, keratin 19 expressing cells display very little proliferative potential. Expression of specific epitopes of a polymorphic epithelial mucin (PEM) is localized to luminal cells in vivo. Cells in the resting gland are weakly PEM positive, whereas cells from lactating glands may express higher levels of specific mucin epitopes. Like keratin 19, high expression of specific PEM epitopes has been correlated with a low proliferative potential in milk derived HMEC in vitro. Only a small fraction (~3-10%) of normal HMEC in vivo show detectable estrogen receptor, and this positive population is preferentially localized in the non-basal layer. It is not clear that the mammary gland contains cells which are terminally differentiated, such as those in the most mature layers of stratified epithelium, since even keratin 19, PEM positive cells have a limited capacity for cell division in vitro.

Unlike what one might intuitively expect, and unlike stratified epithelial tissues, breast tumor cells in vivo and tumor cell lines in vitro almost all have the phenotype of the most mature luminal cell - positive for keratins 19, 8/18, high expression of several PEM epitopes, including those found in the differentiated lactating cells, negative for keratins 5/14, and CLP. The consistency of tumor expression of keratin 19 has been utilized to locate micrometastases in lymph nodes. As normal HMEC with this phenotype show little or no growth in culture, I think the presence of this phenotype in growing tumor cells is indicative of some aspect of derangement in growth control. Most tumors also initially have high expression of estrogen receptor, and most are initially negative for vimentin expression, although vimentin is seen in a subset of estrogen receptor negative breast tumor cell lines and tissues. I don't know of a definitive explanation for this tumor cell phenotype, nor has the maturation state of the tumor cell precursor in vivo been clearly established. Benign proliferative tumors and some in situ carcinomas contain keratin 19 negative cells, so a keratin 8/18 positive, 19 negative cell could be the precursor of invasive breast tumors. If so, expression of the keratin 19 and high PEM phenotype in invasive tumors may be a consequence of malignant transformation.

In collaboration with others, we have examined the HMEC grown under our culture conditions for expression of the above phenotypic markers. Primary cultures of normal HMEC grown in MCDB 170 and early passage cultures grown in MM are heterogeneous. Some cells have the basal phenotype: keratin 5/14 positive, PEM negative, and vimentin, CLP and alpha-actin positive; other cells show the luminal phenotype: keratin 5/14 negative, keratin 8/18/19 positive, PEM positive; and some are in-between (e.g., keratins 5/14/8/18 positive). The cells which initially proliferate in MCDB 170 medium have the basal phenotype. However, post-selection cells begin to express some luminal markers, i. e., keratins 8 and 18 and some PEM epitopes. Expression of these luminal properties increases with continued passage in culture, such that the senescent cells uniformly express these markers. At the same time, expression of the basal keratins 5/14, CLP, and vimentin is not lost. We have not detected keratin 19 or estrogen receptor in the post-selection population. All HMEC examined derived from normal tissues have shown a normal karyotype.

The above results led us to propose in 1989 that the cells which display long term growth in the serum-free MCDB 170 represent a multipotent stem cell population initially present in the basal layer of the gland. With increasing time in culture, these cells show a partial differentiation towards the luminal phenotype. However, it is possibile that culture conditions have induced some artifactual phenotypic expression. In particular, growth of cells on impermeable plastic substrates pevents the normal cell-extracellular matrix contacts and precludes the normal development of cellular polarity. **NOTE:** The post-selection cells in MCDB 170 represent a limited phenotype. While they represent a wonderful normal human epithelial finite lifespan cell with which to study questions of growth control, for some experimental purpose, these may not be the best cell types to use. Cells grown in MM type media show the range of in vivo phenotypes. However, they have a much more limited lifespan and we have less of them to distribute. Additionally, although they contain keratin 19 positive cells, these are not the actively dividing cells in the population.

II. In Vitro Transformation of HMEC

II. A. Derivation of Cell Lines 184A1 and 184B5, and Extended Life Cultures (references: Stampfer & Bartley, 1985; Walen & Stampfer, 1989) Index

One of my main goals in developing a HMEC culture system was to use it for in vitro transformation, so that cells from one individual could be compared at different stages of malignant progression. I was interested in using a chemical carcinogen as the agent for transformation because (1) I wanted to induce random errors; (2) there was a lot of data indicating polycyclic aromatic hydrocarbons (PAH) were good inducers of mammary cancer in rodents, and I wondered if the same could be true for humans; (3) chemical carcinogens seemed an easier physical carcinogen to use compared to radiation; (4) we performed a series of experiments indicating that HMEC were very efficient at converting PAH procarcinogens to their active form (see section V. A.).

Three sets of experiments were performed in the early 1980's using primary cultures from normal HMEC specimen 184 organoids. The three separate original cultures had the FreezeDownSymbols: "aleph"(A), "cross"(C), and "birdie"(B). In each case, cells in at least 2 T-25s were treated for 2 or 3 24 hr periods with 1-2 μg/ml of benzo(a)pyrene (a concentration that gave 80% killing) and 2 T-25s were treated as controls. The cells were grown in **MM** medium, in which 184 normally stops growing by 5th passage. We followed the fate of the treated and control cells both in primary culture (how long growth was maintained in the primary T-25 flasks) and after subculture (the primary flasks were partially trypsinized many times, and after some trypsinizations the removed cells plated and passaged until growth ceased).

Figure 3 shows the fate of these cells and indicates the nomenclature we used to identify Extended Life (EL) cultures and the immortally transformed cell lines. We gave a number to each of the subcultures we passaged in experiments C (cross) and B (birdie); i.e., the B1, B2, B3 etc. that you see in the figure. The EL cultures (i.e., treated cells that kept growing after the controls had stopped growing) derived from each of these subcultures were given the alphabetical equivalent to these numbers, e.g., B5 = Be, C2 = Cb. The immortally transformed lines that developed were given the subculture name, i.e., B5 and A1. We have extremely limited frozen stocks for some of these EL cultures. Subsequently, we grew some of these MM derived EL cultures in MCDB 170, which we found permitted growth for an additional 2-5 passages. Still, we have very limited stocks of EL cells so we're hesitant to distribute them. We would consider specific cases of collaboration or mutual interest, so check with me. The EL cells were notable for their heterogeneity with respect to morphology and growth potential (see Figure 4). Growth often followed a punctuated pattern, with outgrowth (lasting 1-5 passages) of individual patches or colonies within non-growing populations. **NOTE:** most, though not all, of the EL cells had morphologies/growth patterns clearly distinct from anything in the untreated populations. I suspect this implies something about changes which have occurred, which could affect cell-cell or cell-matrix interactions.

Click here to see figure 3.

Legend for figure 3: Growth of BaP treated specimen 184 in MM. It was conveniently fortuitous that the FDSs chosen for these three experiments started with the first three letters of the alphabet. When published, I presented them in the order A, B, C so it would look intentionally linear, but the actual order of the experiments was A, C, B and their real names are aleph, cross, birdie. The figure follows the fate of the treated (T) and control (C) cells in primary culture and upon subculture. Since several dishes were plated at each subculture, and if growing, their lineages followed independently, more than one kind of growth pattern could be observed at a given passage level. In experiment 184C, cholera toxin was inadvertantly omitted from the medium until 22 days after seeding (p5 of subculture C1, p4 of C2, p3 of C3).

Click here to see figures 4a-d

Legend for figure 4: Morphology of EL cultures, Giemsa stained.
(A) 184C p8 with mixed growing and non-growing cells throughout the dish;
(B) 184C p6 containing two focal growing areas, one with uniform growth (shown) and one with mixed growing and non-g rowing cells (not shown);
(C) 184B p6 with non-growing and actively growing cells in a "hyperplasia" morphologic pattern;
(D) 184C p7 with swirly thumbprint morphology. 184Aa had almost exactly the same appearance when it first showed up as a single patch in 184A p5.

Eventually, almost every EL cell ceased growth. The two exceptions were the appearance of the 184A1 line from the 9th passage 184Aa EL population, and 184B5 from the 6th passage 184Be. 184A1 stood out as a more refractile appearing cell growing more vigorously as "eye-shaped" singlets, compared to the patchier, flatter, less vigorous 184Aa (which died by passage 11). Some cells were transferred to MCDB 170 medium at passage 11 and carried continuously in that medium to passage 105. Cells were also maintained in MM up to passage 69. 184B5 was a sickly looking small tight patch, somewhat more refractile than 184Be, very slow growing, that strongly caught my attention for undefinable reasons. It was first transferred to MCDB 170 at passage 9 and grown to passage 101. Cells maintained in MM were grown to passage 30. It is curious and

perhaps indicative of some underlying structural change that the first time I saw both these cells, I was sure they were transformed, and I did not have that sense with any other cells in the EL cultures.

Both of these lines show a few specific clonal karyotypic aberrations, indicating their independent origins from a single cell. Some of the karyotypic abnormalities found in 184B5, e.g., 1q22 breaks and tetrasomy for 1q, are also frequently observed in cells obtained from breast tumors. Upon continued passage in culture, these two lines show a little genetic drift (more so in 184B5 than 184A1) , but it is relatively minimal compared to that observed in most human breast tumor cell lines. Even at passage 41, 184B5 has clearly identifiable chromosomes and a near pseudodiploid karyotype. Thus, the vast majority of the cell population would be expected to remain karyotypically stable when studied over the course of a few passages in culture, yet the presence of some genetic drift could give rise to rare variants in the cell population. Although 184A1 and 184B5 have an indefinite lifespan, they do not have properties associated with malignant transformation. They do not form tumors in nude mice and th ey show very little (184B5) or no (184A1) capacity for anchorage independent growth (AIG). 184B5 has a distinctive morphology, growing in tightly packed patches. An advantage of this is that the fate of single cells can easily be followed without needing to seed at clonal densities (the progeny of a single cell stay attached and make a colony). At passages <30, 184A1 cells tended to grow with minimal cell-cell contact when cultures were not dense, and showed some morphologic heterogeneity, with the presence of large vacuolated cells. At >30 passages a growth pattern showing more cell-cell association and patchiness started to appear, and fewer vacuolated cells were visible (see Figure 5 and discussion in section IV.).

Click here to see figures 5a-d

Legend for figure 5: Morphology of 184A1 and 184B5, Giemsa stained. All pictures shown at the same magnification.
(A) 184 p9 in MCDB 170;
(B) 184A1 p15 in MCDB 170; note the large vacuolated cells;
(C) 184A1 p42 in MCDB 170;
(D) 184B5 p11 in MCDB 170.

Since 184A1 and 184B5 are cell lines of indefinite lifespan, I have unlimited supplies to distribute. However, more recent studies have shown significant changes in these populations with continued passage and from clone to clone, so this may need to be considered (see section IV.). Anyone who has used these cells previously may want to check in with me on this.

CAUTIONARY NOTE: We were remiss in our earliest tissue culture years in not routinely checking all cells for PPLO contamination. We first started routinely testing for PPLO in 1982, after experiments "A", "C" and "B" were initiated, and the cell lines 184A1 and 184B5 were being maintained in MCDB 170. These lines, as well as other normal and benzo(a)pyrene treated extended life cells growing in MCDB 170, were tested for PPLO by Hoechst stain and growth in agar broth. The results were all negative. About a year later, we took out some of our frozen extended life benzo(a)pyrene treated cells, and placed them in MM. Now our routine Hoechst stain test showed some of them to have foreign DNA, although broth growth was still negative. Transfer of the samples to MCDB 170 generally led to loss of the Hoechst stain positive material within 2 passages. This negative phenotype was retained after transfer back to MM. Two (visually equivalent Hoechst stain positive) samples were sent to Microbiologic al Associates for assay by agar growth, Hoechst, and strain-specific antibodies. They reported one to be completely negative and the other positive for M.hyorhinis (which doesn't grow in the usual agar broth assay). From our Hoechst stain results, it appears that 184Aa, the EL precursor of 184A1, was positive, while some, but not all of 184Be, the EL precursor of 184B5, may have been positive. We can not say whether this may, or may not, have affected any results. By the Hoechst and Micotect assay, our current normal HMEC, EL, 184A1, and 184B5 are negative. It was a mistake for us not to have been testing our cells. A take-home message is to be careful to check your cells on a regular basis.

II. B. Derivation of Variants of 184A1 and 184B5 (references Clark et al., 1988; Stampfer & Yaswen, 1992) Index

One of my original goals for in vitro transformation of HMEC was to obtain malignant transformants from normal HMEC. To be honest, somewhere along the line I realized that although this was important science, I myself was less than enthusiastic about creating malignant cells from normal, and the immortally transformed cells were sufficient for my scientific curiosity (or as I commented, as I got older, the question of immortality seemed more interesting than malignancy). Nonetheless, I did try (and not succeed) to obtain malignancy by exposing the 184A1 and 184B5 lines to further chemical carcinogens, in this case, the direct acting carcinogen, N-nitroso-ethyl-urea (ENU). Others exposed the lines to specific oncogenes, which could lead to cells that

were AIG and/or made tumors in nude mice. As part of this work, I analyzed the cells for their nutritional requirements and isolated/developed cell variants with altered nutritional requirements. The history of these studies and the names/origin of these variants is described below.

II. B. 1. Nutritional Variants

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Nomenclature Note: Where spontaneously occurring subpopulations were isolated based on the nutritional composition of the medium, they are designated as **A1N...** and **B5N....** In the case of MM grown subpopulations, the N is followed by a (arbitrary) number; in the case of MCDB170 grown cells, the N is followed by letters indicating what factors were no longer required. Nutritional variants obtained following exposure to ENU are designated **A1ZN...** and **B5ZN...** followed by letters indicating the non-required factors. While I sometimes refer to these nutritional variants by their complete names (e.g.., 184A1N4), for brevity and simplicity sake, it's OK to officially refer to them without the 184 prefix.

The first nutritional variants were isolated (in a none too systematic fashion) from 184A1 growing in MM medium. For general purposes, the only one of these to note is **184A1N4.** 184A1 was seeded at p16 in MM minus the conditioned media and without cholera toxin. Attachment appeared poor but the few patches that were present grew fairly well. After 3 passages, there appeared to be uniform good growth and attachment. These cells were first transferred to MCDB 170 at p28. The karyology of A1N4 indicated that, unlike the pseudodiploid 184A1, A1N4 were anueploid (near triploid) with only one additional chromosomal marker beyond the 4 seen in the parental 184A1 cells. It is therefore likely that, although not cloned, they represent a clonal population. The A1N4 were used by Robin Clark for malignant transformation with oncogenes.

More systematic isolation of nutritional variants was done in MCDB 170 medium. The existing literature indicates that many transformed cells show reduced nutritional requirements. As part of our initial characterizations of the immortally transformed cell lines, we first compared the requirements of 184, 184A1, and 184B5 for the individual growth factors present in MCDB 170 for short term growth, for long-term culture, and in clonal vs. mass culture (Table 2 and list below). 184A1 and 184B5 showed afew differences from each other and normal HMEC. Both were more dependent upon EGF for growth in mass culture (although EGF independent variants could be isolated) whereas the normal cells could continue to proliferate without EGF (see section III.). A ll of these HMEC showed a stringent requirement for EGF in clonal culture. 184A1 showed little effect upon removal of hydrocortisone (HC); 184B5 and 184 had greater short-term requirements. All the HMEC had a requirement for BPE for short-term growth. In the long-term experiments, removal of HC or BPE from mass cultures of normal HMEC led to cessation of growth over the course of 1 to 3 passages. Removal of insulin (I) did not prevent continued proliferation, but led to slower growth, a less healthy appearing culture, and earlier senescence. Removal of I from 184A1 and 184B5 also did not prevent continued growth. For more details of the long-term experiments with the cell lines, see the list below. These nutritional variants are available for distribution.

Table 2: Growth Factor Requirements of Normal and Transformed HMEC in MCDB170

	Percentage of Control Cell Growth					
	184		184A1		184B5	
Medium	**MC**	**CFE**	**MC**	**CFE**	**MC**	**CFE**
Complete MCDB 170+IP	100	100	100	100	100	100
minus I	49	47	11	18	26	73
minus HC	36	32	84	88	18	61
minus EGF	86	2	20	0	12	0
minus BPE	15	21	21	24	16	75

Abbreviations used: **I**, insulin; **HC**, hydrocortisone; **EGF**, epidermal growth factor; **BPE**, bovine pituitary extract; **IP**, isoproterenol; **MC**, mass culture growth; **CFE**, colony forming efficiency.
Cells from specimen 184 (p11), and cell lines 184A1 and 184B5 (passages 17-20) were grown in complete

MCDB 170 with isoproterenol. For mass culture, cells were subcultured into duplicate 35 mm dishes (5 x 104 per dish) in the indicated media. When control cultures were subconfluent or just confluent, all the cultures were trypsinized and the cells counted by hemocytometer. For clonal cultures, single cells (100-1000) were seeded into triplicate 100 mm dishes. After 10-14 days, cells were stained with Giemsa and colonies greater than 30 cells counted.

List of Spontaneous Nutritional Variants: 184A1: CAUTION: the nutritional requirement studies were done with 184A1 at passages <20 and the selection for variants was done with 184A1 around passages 27-32. We now know that these are non-homogeneous populations (see section IV.) The results might be different if this variability were considered.

184A1NE: no EGF. For the first 2 passages, growth was slow and selective (a small # of patches). The growth rate was the same as control (+EGF) after 4 passages.
184A1NH: no HC. Slow patchy growth for first passage; growth normal after 2 passages.
184A1NI: no I. Growth was initially slowed, but less selective than -EGF; the cells looked good. Growth rates were normal within 2-4 passages.
184A1NB: no BPE. Little initial growth. Eventually a few patches grew out. After one additional passage the resultant cells grew normally.

184B5:

184B5NE: no EGF. Media first changed at p48. Growth initially slower and selective. It took 7 passages to select a population that looked good and had a normal growth rate. Repeated with cells at p36, after 3 passages of slow, selective grow th a good growing population arose.
184B5NH: no HC. Media changed at p34. Growth initially slower but normal after 2 passages.
184B5NI: no I. Media changed at p35. Growth initially slowed but not as selective as -EGF. Growth rates were normal within 4-6 passages.
184B5NB: no BPE. Growth initially slow and selective but not as extreme as 184A1 -BPE. Cells didn't look good and grow normally until after 6 passages.
184B5NIB: no I or BPE. 184B5NB cells were switched at p42 to media without insulin. They grew initially slowly and poorly. Good patches were obvious after 2 passages and growth was normal after 3 passages.

We next examined the effect of removal of multiple growth factors to determine conditions where untreated 184A1 and 184B5 did not yield nutritional variants. Such conditions could then be used to select for ENU induced variants. These conditions were defined as removal of I and EGF, I and BPE, or EGF and BPE for 184A1, and removal of I and EGF, or I and BPE for 184B5. Populations of 184A1 and 184B5 were then tested for their ability to grow in these restrictive media after exposure to ENU concentrations that yielded 80% inhibition of colony forming efficiency (1500 µg/ml for 184A1 and 750 µg/ml for 184B5). Two T-75 flasks each of treated and control cells were exposed to ENU or solvent alone for 2 or 3 consecutive passages. Under a few conditions the ENU treated cells were capable of sustained growth whereas the untreated cell lines quickly ceased growth. The resulting growth factor independent variants may represent a further step in malignant progression. However, they did not show AIG or form tumors in nude mice.

List of ENU-induced Nutritional Variants:

184A1ZNEB: selected in MCDB170 -EGF-BPE. The treated cells had a fair amount of growth (compared to almost nothing in the controls), but most of this faded away after several passages. In one experiment, cells with patchy vigorous growth and a distinctive morphology quickly took over the population, and maintained active growth in this medium. Although not examined, these are presumably clonal.
184B5ZNEI: selected in MCDB170 -EGF-I. The treated cells showed initial widespread, morphologically heterogeneous, growth (compared to very little in the controls). Most of this growth faded after about 5 subcultures but in several cultures growth was maintained. The morphologies are not particularly distinctive and we don't know if these represent clonal cultures.

These variants are available for distribution. For more information on the non-EGF requiring variants, see Figure 7.

II. B. 2. Oncogene Exposed Derivatives
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AIG and malignant derivatives of 184A1 and 184B5 have been obtained with the use of oncogene containing retroviral vectors and viruses, and transfection. In the case of 184A1, A1N4, a clonal derivative with reduced nutritional requirements, was exposed by Robin Clark to the genes for SV40 large T antigen, v-H-ras, and v-mos, singly and in combination. The combination of H-ras and SV40-T led to cells (designated **A1N4-TH**) which formed progressively growing tumors in nude mice and showed AIG. Ex posure to v-H-ras (**A1N4-H**) or v-mos (**A1N4-M**) alone led to cells that produced tumors with reduced frequency and longer latency. SV40-T alone (**A1N4-T**) did not yield tumorigenic cells, but did affect the growth factor requirements for anchorage dependent and independent growth. In all cases of oncogene exposure, the resultant cells were capable of proliferation in media that did not support the growth of the parental A1N4 cells. A1N4-TH has a near tetraploid karyotype, which is missing the A1N4 chromosomal marker and contains only one additional clonal chromosomal aberration relative to 184A1. Thus even the malignantly transformed cell line, containing v-H-ras, does not show a very unstable karyotype in terms of gross chromosomal aberrations.

The 184B5 cell line was exposed to v-K-ras (designated **184B5-K**) by Paul Arnstein, yielding cells which were 100% tumorigenic in nude mice, with short latency. However, these tumors did not grow beyond approximately 5 cm diameter. Most of our studies have utilized the culture designated **184B5-KTu**, which was derived from a B5-K tumor resected from a nude mouse and replaced in culture. B5-K and B5KTu do not display AIG.

We have some stocks of these cells available, but we are not eager to be growing or distributing these cells, as the infection was with non-defective retroviruses. If you really want oncogene exposed 184A1 or 184B5, I suggest you transfect the cells with the oncogenes of your choice. Alternatively, other investigators may have already done so, and you may query on this bulletin board for transfected cell lines.

184B5 has also been exposed to transfection with erbB-2, mutated erbB-2, and the insulin receptor. ErbB-2 alone (**184B5-E**) made the cells capable of AIG (~5-15%, large colonies), while mutated erbB-2 additionally made them capable of tumor formation in nude mice. Overexpression of the insulin receptor also made the cells capable of some AIG. We have our stocks of 184B5-E. 184B5 (grown in a serum containing medium) transfected with erbB-2 or with mutated erbB-2 were made by Jaquelyn Pierce and Stuart Aaronson.

II. C. Characterization of 184A1, 184B5, and derivatives (references: Stampfer & Bartley, 1985; Stampfer & Yaswen, 1992; Stampfer & Yaswen, 1993; Sanford et al. 1992; Lehman et al. 1993; Thompson et al, 1994)
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In general, 184A1 and 184B5 have a somewhat more mature phenotype than the normal 184 HMEC in culture. However, it is important to recall that these lines were derived from cells grown in MM medium, which contains cells with phenotypes more mature than basal. We don't know the cell of origin of the EL 184Aa and 184Be precursors to these lines. Both lines maintain some expression of keratins 5 and 14, but at significantly decreased levels, while expression of keratin 18 is increased relative to norma l post-selection 184 HMEC. Both lines have barely detectable levels of vimentin. 184B5 strongly expresses the luminal and tumor associated PEM antigens, while 184A1 has some but lower expression of PEM. The tumorigenic transformants, A1N4-TH and B5-KTu have very low levels of keratin 5 and increased levels of keratin 18. While B5-KTu remains vimentin negative, the A1N4-TH cells show re-expression of vimentin. We have not been able to detect keratin 19 or estrogen receptor in any of these lines. Thus none of these lines fully resembles the tumor cell in vivo, and the phenotypic differences between the immortal lines and MCDB 170 grown 184 HMEC may just reflect maturation states (i.e., not be related to the immortalization process). Like normal proliferative HMEC, they are keratin 19 negative.

Fibronectin represents about 10-20% of the protein secreted by normal HMEC in culture. In many transformed cells, the level of fibronectin mRNA and protein synthesis is decreased. Expression of fibronectin is greatly reduced in 184A1 and to a lesser extent in 184B5. However, we do not know if fibronectin secretion would normally be lower in HMEC with a more mature phenotype. Upregulation of fibronectin synthesis by TGFb remains normal in both cell lines (see section IV.).

The normal and transformed HMEC have also been characterized with respect to both their growth patterns and their gene expression when placed on reconstituted basement membrane material derived from the Englebreth-Holm-Swarm (EHS) murine sarcoma, which has been shown to support increased differentiated functions of a variety of cell types. Normal HMEC are capable of forming three-dimensional structures with striking resemblance to endbuds in intact mammary gland tissue, whereas 184A1 displays only less developed

structures and 184B5 forms only small clusters. The A1N4-TH cells show even less structure formation than 184A1 and the B5-KTu cells resemble 184B5. We have not examined the underlying basis for these differences, and suspect that alterations in cell-cell connections may be involved. E-Cadherin is expressed by all of these cells with the exception of the aggressively tumorigenic A1N4-TH cells.

As mentioned earlier, 184A1 and 184B5 differ from normal HMEC in having some karyotypic abnormalities. 184B5 has been shown to have a 10x higher rate of mutations at the HPRT locus than normal HMEC, reduced intercellular communication, and reduced DNA repair during the G2 phase. The ability of 184A1 and 184B5 to gain AIG and to be malignantly transformed with specific oncogenes also differs from normal HMEC.

No differences in sequence or expression of p53, or p53 dependent genes, has been seen in 184A1 or 184B5 relative to normal HMEC. However, we and others have shown that the p53 expressed by these cultured HMEC (but not the p53 in cultured fibroblasts from the same person) is in a conformation recognized by antibodies that recognize mutant p53, and the half-life of the p53 protein is 3-4 hrs - longer than what has been seen for fibroblasts or rodent derived epithelial cells.

Another approach we took to characterize differences between our normal and transformed HMEC cultures was to use subtractive hybridization to identify genes expressed in the normal HMEC, but downregulated in the immortal and malignantly transformed cells. This was how we first isolated and identified CLP (see section V. B. for more), and observed the difference in expression of keratin 5, vimentin, and fibronectin. **NOTE:** 184A1 and 184B5 are immortally transformed cell lines. Normal HMEC have a finite lifespan; immortality is not normal. Therefore, I think it grossly incorrect to refer to these lines, or any immortal cell line, as "normal",. even though they do retain many normal properties. A highly significant aspect of these cells is not normal.

III. Synchronization of HMEC Cultures and Role of EGF Receptor Signal Transduction

(references: Stampfer, Pan et al. 1993; Stampfer and Yaswen, 1993; Bates et al., 1990)
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One of the my long-term goals in developing the HMEC system was to ask questions related to mechanisms of growth control, such as those controlling expression of finite lifespan, senescence, escape from senescence (immortality), and the role of specific positive and negative growth factors in normal and transformed cells. I assumed that these processes would be connected to cell cycle control, and that in order to examine the cell cycle, it would be necessary to obtain synchronized cell populations. Therefore, I looked for a method to synchronize the HMEC. Additionally, I wanted to find a method that would not involve use of metabolic inhibitors or general starvation, and thus potentially not be cytotoxic or stress inducing.

We had previously shown a stringent requirement for EGF receptor (EGFR) ligands (e.g., EGF/TGFa) for clonal growth of the HMEC, although the normal HMEC could grow in mass culture without addition of exogenous EGF. Further study demonstrated that the mass culture growth was due to an autocrine loop resulting from endogenous production of EGF like ligands such as TGFa and amphiregulin. Blockage of EGFR signal transduction with an antibody to the EGFR prevented growth. Although 184A1 and 184B5 synthesized similar amounts of TGFa as normal 184 HMEC, they failed to secrete this protein, and thus required addition of EGF to the medium for growth (see figure).

The above data suggested that blockage of EGFR signal transduction might provide a method to reversibly arrest these HMEC, and this possibility was examined in detail. After growth with EGF to midconfluence, HMEC cultures were maintained for 48 hrs in medium without EGF and containing monoclonal antibody (MAb) 225 to the EGFR. Cells were then refed with medium containing 5 times normal EGF. During exposure to MAb 225, the HMEC acquired a less refractile morphology with increased cell-cell contact, decreased motility, and few mitoses. After re-exposure to EGF, the typical cobblestone epithelial morphology and many mitoses were visible by 24 hrs. Normal HMEC could be maintained for at least 18 days in EGF deficient medium plus MAb 225 and still regain a normal cobblestone appearance with many mitoses after EGF re-exposure, suggesting that the growth inhibited cells were arrested in a viable, non-cytotoxic state (I refer this state as sleeping/hibernating). We have not systematically tested 184A1 and 184B5 for long-term viability in minus EGF medium; they may differ from the normal HMEC in their ability to "hibernate".

Protein and DNA synthesis in normal 184 and 184B5 were assayed by incorporation of 14C-leucine and 3H-thymidine (Figure 6A&B). Protein synthesis remained depressed as long as the antibody was present and increased rapidly following re-exposure to EGF. DNA synthesis decreased 12 hr after antibody addition, and

was sharply decreased by 24 hr. DNA synthesis resumed only 10 hr after EGF re-exposure and then increased sharply to a peak around 18 hr. We have examined cells from reduction mammoplasty specimens 48 and 161, and found similar results. For specimen 48, DNA synthesis following restimulation with EGF began and peaked about two hours earlier, and there was greater synchrony exiting S phase, suggesting that good synchrony could be maintained into the next cell cycle.

Click here to see figure 6a Click here to see figure 6b

Legend for figure 6: Effects of blockage of EGF receptor signal transduction on DNA and protein synthesis by normal 184 HMEC and 184B5.
(A) Cells from specimen 184 were grown in 35mm dishes in complete MCDB 170 until midconfluence. Treated cultures were then exposed to MCDB 170 minus EGF plus 8 μg/ml MAb 225 for 49hr, while control cultures received complete MCDB 170. After 49hrs, all dishes were washed once with PBS and refed. Treated cultures were refed with either complete MCDB 170 containing 25 ng/ml EGF (Triangle) or maintained in MCDB 170 minus EGF plus 8 ug/ml MAb225 (square). Control cultures (circle) were refed with complete MCDB 170 containing 25 ng/ml EGF. Cells were exposed to a 2 hr pulse of 5 μCi 3H-thymidine (closed symbols) and 80 nCi 14C-leucine (open symbols) in 1.5ml of medium for 1 hr before and after the indicated times. Total acid-insoluble counts were then determined and are presented on a per dish basis.
(B) Cells from 184B5 were treated as for 184 with two differences: no Mab 225 was used and the cells were kept -EGF for 48 hrs.

Click here to see figure 7.

Legend for figure 7: TGFa production and secretion, and effects of EGF on growth and DNA synthesis of normal, immortally transformed, and EGF independent variant HMEC. To determine the effect of EGF on growth rates, 0.5 x 10^-5 cells were seeded into 35mm dishes in either complete MCDB 170, MCDB 170 minus EGF, or MCDB 170 minus EGF containing 6 μ/ml of MAb 225. The number of attached cells was determined 16-24 hr later. When control cultures (complete MCDB170) were just confluent, all cell cultures were trypsinized and cell numbers determined by Coulter Counter. To determine the effect of EGF on DNA synthesis, midconfluent cultures that had been grown in complete medium were switched to the indicated medium for 24 hr. For the last 2 hr, cells were exposed to 4 μCi 3H-thymidine in 1.5 ml. Acid precipitable counts were determined by scintillation counting. To determine TGFa synthesis and secretion, cells were grown in 60 mm dishes until subconfluence. 24 hr conditioned medium was then removed, and the cells harvested and frozen. Radioimmunoassays of medium and cells were performed by Robert Coffey, Vanderbilt University. These data indicate that 184A1 and 184B5 synthesized amounts of TGFa similar to normal 184 HMEC, but failed to secrete this protein. The ability of the variant lines to maintain growth in the absence of EGF did not appear to be due to increased TGFa secretion. Although A1NE and B5NE could continue to grow in the absence of EGF, their rate of growth was decreased, and they were still sensitive to MAb 225 induced inhibition of growth and DNA synthesis. It is possible that increased synthesis of another ligand for the EGF receptor, such as amphiregulin, may be responsible for their altered growth properties. The ENU induced variant, A1Z NEB also grew faster in the presence of EGF, but it was no longer sensitive to MAb 225 inhibition. Additionally, it showed significantly increased levels of cell-associated TGFa protein. One possible explanation for this phenotype is internal or membrane associated stimulation of the EGF receptor. The ENU induced variant B5ZNEI showed no difference in growth rate with or without EGF, but was still partially sensitive to MAb 225 inhibition.

Specimen 184 was tested for how long a period of EGF re-exposure was needed following arrest to allow cells to subsequently enter S phase, with the result that a 1 hr exposure was sufficient to allow the majority of cells capable of cycling to later enter S. Thus EGF seems necessary just to get the cells into cycle (a competence rather than a progression factor). All the other growth factors were present in the medium, so we can not say if any of them specifically function as progression factors.

The above results suggested that HMEC restimulated with EGF following the growth arrest were exiting a Go state and entering S phase in a highly synchronous fashion. Examination of early response gene expression supported this conclusion. Expression of c-myc, c-jun and c-fos was readily detectable in normal cycling HMEC cells, but decreased during growth arrest. High levels of mRNA for all these genes were observed at 1 hr following re-exposure to EGF. 184B5 differed from normal HMEC in not showing any decrease in expression of the early response genes during the Go arrest, while 184A1 at p31 showed a partial decrease. Synthesis of TGFa mRNA, which was also inhibited in the presence of MAb 225, was detected by 2 hr after EGF re-exposure. Some mRNA species, such as for keratin 5 and CLP, continued to be expressed during the growth arrest.

Studies done largely with growth arrested fibroblast cells have defined a Go state characterized by low metabolic activity, a rapid increase in levels of mRNAs for certain early response genes upon release from the growth arrest, and an increase of 6-7 hr in the time required to begin DNA synthesis following release from growth arrest, relative to continuously cycling cells. These properties are all observed with the HMEC growth arrested by EGFR blockage, suggesting that this is a Go arrest. Thus, blockage of EGFR signal transduction is sufficient by itself to cause normal and immortally transformed HMEC to enter a Go-like resting state. Unlike fibroblast cells from the same specimens, the levels of c-myc mRNA in HMEC remained high throughout the cell cycle, while c-fos and c-jun mRNA, though showing cycle dependent fluctuation, were readily detectable in the cycling epithelial cell populations.

Exit from a Go state is not the same as passage from M into G1. In experiments with 184B5, we examined the mRNA synthesis into the second cycle, and could see an absence of the strong burst of early response gene expression as cells entered G1 without a Go exit. Levels of these mRNA species did increase, and it could be seen that the rise in c-fos expression preceeded that for c-myc and c-jun. To study the cycle without a Go arrest, one can follow the cells into the second cycle, or use a method for G1 arrest developed by Keysomarsi et al., 1990, which utilizes Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

IV. Effects of TGFb on Normal and Transformed HMEC (references: Hosobuchi & Stampfer, 1989; Stampfer, Yaswen et al. 1993)
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TGFb is a pleotropic cytokine known to be a potent growth inhibitor of normal epithelial cells in vitro and in vivo. It modulates several key physiologic processes such as wound healing, differentiation, and tissue morphogenesis and remodeling, and is also thought to be involved in carcinogenic progression. Varying degrees of resistance to TGFb induced growth inhibition are seen in human carcinoma cells, and this loss of negative growth regulation may contribute to tumor development. While in some instances, resistance correlates with loss of functional type I and II TGFb receptors, most resistant tumor lines express normal numbers of apparently functional receptors.

I initially examined the effects of TGFb on HMEC to determine if it could be a good method for cell synchronization. As will be described, this was clearly not the case. However, in the course of these studies I kept coming up against what seemed to me as really strange results, and I have been trying to track down the bases of these oddities ever since. It's been an intriguing puzzle, and perhaps I finally have some sense of the picture, but details and mechanism are still fuzzy.

My first difficulty with TGFb was simply getting consistent results on the extent and speed of growth inhibition of normal HMEC from the same individual. This mystery was partially solved when I controlled for passage level and batch. The effect of TGFb on some individual specimens depended upon age in vitro. While every finite lifespan HMEC that we have tested is ultimately growth inhibited by TGFb, younger cells in culture may undergo 8 or more population doublings before full arrest, where as older cells stop growth in 1-2 population doublings, and with lower TGFb concentrations (see figure 8). The normal HMEC show distinctive morphologic changes in the presence of TGFb, characterized by an elongated, flattened appearance. The growth inhibition was only partially reversible, the extent of reversibility decreasing with age in vitro, and was relatively asynchronous (see figure 9A). The cells were not in a resting state, since 14C-leucine incorporation indicated that protein synthesis was stimulated even as growth was inhibited (see figure 9A). More recent studies using 3H-thymidine incorporation have indicated that the growth arrest is in mid to late G1; TGFb added at >= 10 hrs following Go exit was not growth inhibitory.

Click here to see figure 8.

Legend for figure 8: Effect of TGFb on growth of normal and transformed HMEC. Cultures were seeded into triplicate 35 mm dishes (4-5 x 10-4 cells/dish) in the indicated concentration of human recombinant TGFb1. The number of attached cells was determine d 4-16 hr later. When control cultures were subconfluent or just confluent, all cell cultures were trypsinized and cell numbers determined by Coulter Counter. The attached cell number was subtracted from the final cell count. Data are presented as percentage cell number of the TGFb exposed cells relative to the non-TGFb treated controls. NOTE: When I refer to the finite lifespan cells as TGFb sensitive, this means that no cell (as in zero) has been capable of maintaining growth in the presence of TGFb (although "younger" cells can take a week or two before all growth stops). This is very different from what may be called 'TGFb sensitive" breast tumor cell lines, where what is referred to is often a reduction in cell number or growth rate. When I refer to immortally transformed cells as TGFb resistant, this means that the cells can maintain growth indefinitely in the presence of TGFb. However, there

may still be some reduction in growth rate, or cells which don't maintain growth.

Another mystery which I never solved (or published, since I didn't know what to make of it) was stumbling across the observation that addition of MAb 225 to normal HMEC growth arrested by TGFb led to synchronous entry into S phase, within 3 hrs, of the cell population that was reversibly inhibited (see figure 9A&B). This seemed to imply that some function of the EGFR was required to maintain TGFb growth arrest in late G1.

Click here to see figures 9a. Click here to see figures 9b.

Legend for figure 9: Effect of addition of MAb 225 on cells arrested in late G1 by TGFb.
(A) 184 p12 were seeded in triplicate 35mm dishes in MCDB 170 until midconfluence. Treated cultures (square) were then exposed to 5ng/ml TGFb for 48 hr. All dishes were then washed once with PBS+0.1% BSA. The TGFb treated cultures were refed with either complete MCDB 170+BSA with no TGFb (triange), MCDB 170+BSA with 6 µg/ml MAb 225 (diamond), or maintained in MCDB 170+BSA+5ng/ml TGFb (square). Control cultures (circle) were refed with complete MCDB 170+BSA. Cell labeling was as described in Fig. 6. Results are presented on a per dish basis. Cells exposed to continuous TGFb had about 1/2 the cell number as control cultures after 48 hr, and about 1/3 after 72 hr.
(B) 184 p13 were seeded into triplicate 35 mm dishes and grown in MCDB 170 until sparse- midconfluent. Treated cultures were then exposed to 20 ng/ml TGFb for 48 hr. All dishes were then washed once and refed as indicated. Cell labeling was as described in Fig. 6. Results are presented on a per dish basis. Cells exposed to continuous TGFb had 1/3 the cell number as control dishes after 72 hr of TGFb exposure. TAb 1 is an antibody to TGFa. It's effect was generally similar to that of MAB 225, indicating that the observed result with MAb 225 is not due to an agonist effect; however, since EGFR ligands in addition to TGFa may be present, TAb 1 did not have as stringent an effect as MAb 225.

Examination of 184A1 and 184B5 for their responses to TGFb is what really caught my attention. Whereas I had never seen a single finite lifespan HMEC maintain growth in TGFb, the immortally transformed HMEC lines could give rise to populations that maintained growth indefinitely in the presence of TGFb. Initial puzzles:

When mass cultures of 184B5 were examined around passages 26-30, they appeared to maintain active proliferation in TGFb. However, when I looked at clones isolated at early passages, they appeared much more growth inhibited. One clone, designated 184B5T1 (isolated at p13) was extremely growth inhibited. It also had the strange property that on four separate occasions, almost all the cells ceased proliferation, looked gross (that's what we wrote in our notes) and died around passage 30. A few (around 1 in 10-5) cells survived this "crisis" and these cells were now capable of maintaining growth in TGFb. More careful observation, utilizing the clonal growth characteristic of 184B5, indicated that many 184B5 cells seemed to be dying, i.e., at all early passages there were many small colonies that never progressed and consisted of big flat yukky-looking cells.

Mass cultures of 184A1 were first examined around passages 27-39. They appeared to be largely growth inhibited by TGFb. However, a small number of cells maintained good growth indefinitely. I assumed these represented rare mutants, and therefore isolated 4 single cells clones to get pure populations. When these were grown up and examined, they all gave the same results as the uncloned line - most cells were growth inhibited and a few maintained good growth. I hadn't a clue how to explain this (this was 1988), and couldn't get others interested in this phenomena. It seemed like the cell biologists knew something was fishy about TGFb resistance, but didn't want to talk about it publicly, and the molecular biologists were too busy looking for **the** mutation which conferred resistance.

Although growth responses to TGFb varied among the normal and immortalized HMEC, all of these HMEC showed a similar profile of TGFb1 receptors and all expressed specialized responses to TGFb including strong induction of: mRNA and/or protein for extracellular matrix associated proteins such as fibronectin, collagen IV, and laminin; the proteases, type IV collagenase and urokinase type plasminogen activator; the protease inhibitor, plasminogen activator inhibitor 1. The level of overall protein synthesis, especially secreted proteins, was increased following TGFb exposure even where cell growth was inhibited. These results indicated that the effects of TGFb on HMEC proliferation could be dissociated from its effects on specialized responses likely to play a role in glandular remodeling, homeostasis and/or wound healing. It should be noted that fibroblast cells can show similar specialized responses to TGFb in the absence of growth inhibition. Fibroblasts from specimen 184 show a slight growth stimulation in TGFb.

The plasticity in resistance to TGFb growth inhibition by these immortal HMEC lines was difficult to explain by any simple mutational mechanism; we originally suggested that epigenetic, as well as genetic mechanisms,

may be involved Additionally, the apparent correlation between the capacity to express indefinite growth potential and the capacity to maintain growth in the presence of TGFb led us to consider whether these two processes might be functionally related in these HMEC. Experiments currently in progress suggest that this might be the case. *(to be continued, experiments in progress - brief preview below for consideration in experimental protocols past and future)*

Examination of these various cell types for expression of telomerase activity and telomere length indicates thus far a strong correlation between expression of TGFb resistance and expression of telomerase activity. Thus, the earliest passage 184A1 cells do not display detectable telomerase activity using the sensitive PCR-based TRAP assay and their telomeres continue to shorten with cell division. With increasing passage, both telomerase activity and resistance to TGFb become increasingly detectable. Populations selected for their ability to grow well in TGFb show high telomerase activity. No obvious crisis is observed in the 184A1 population as a whole, although seeding cells at clonal densities indicates that most cells do not maintain growth even in the absence of TGFb. Early passage 184B5 populations have detectable telomerase activity, however the clones which are highly sensitive to TGFb growth inhibition, and eventually crisis or die, show barely detectable or no telomerase activity. In contrast, clones that show some initial resistance to TGFb growth inhibition, and the TGFb resistant cells which survive crisis, have some telomerase activity. Further studies are underway to provide mechanistic explanations for these data. I venture the following tentative model:

Survival of crisis in the extended life 184Aa and 184Be cultures leads to "conditionally immortal" cell lines, the 184A1 and 184B5. These cells presumably harbor a mutation which permits continued viability and growth past crisis, and is permissive for telomerase activity without additional mutations, but most or all of these cells do not immediately express telomerase activity. Some other event, which appears to be epigenetic, gradual, and may occur only after the telomeres have become critically shortened (mean TRF <2.0 kb), is necessary for reactivation of telomerase activity. This "conversion" event might involve changes in heterochromatin conformation and transcriptional activity. Although most cells probably do not survive to undergo "conversion", the frequency of "conversion" is sufficiently high and occurs over an extended period of time, so no massive cell death is readily apparent in the uncloned 184A1 and 184B5 populations. However, the big vacuolated cells that I saw in 184A1 earlier passages and the non-growing 184B5 colonies appear to represent dying cells which have not reactivated telomerase activty.

V. Other Properties of HMEC System

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Of the variety of studies we and others have done on these HMEC cultures, I want to incude two topics in this review that, at various times, have been the subject of extensive investigation, and may be relevant to work of others.

V.A. Metabolism of Chemical Carcinogens

(references: Stampfer et al., 1981; Bartley at al., 1982; Bartley and Stampfer, 1985, Leadon et al., 1988)

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Before we began the studies on BaP transformation of normal HMEC, we had performed extensive studies on the capacity of human mammary epithelial and fibroblastic cells to metabolize the PAH class of procarcinogens. PAH like BaP require a series of metabolic steps for conversion of the inactive procarcinogen into the active, ultimate carcinogenic form, the diol-epoxide, which is capable of forming bulky adducts with DNA. Since the extent and pattern of BaP metabolism can vary greatly among species, as well as among different individuals and cell type within one specie, we examined the rate and path of BaP metabolism in cells from many indivduals.

Our results indicated that HMEC are extrememly active in metabolizing BaP through the pathways that lead to the 7,8-diol-9,10-epoxide, which can form adducts with the deoxyguanosine of DNA. in contrast, the same concentration of BaP given to fibroblast cells from the same person's breast tissue yielded a much slower rate of metabolism, mainly through pathways that do not lead to the diol-epoxide, and a much lower production of DNA adducts. At the time these studies were performed, it was surprising to observe this high degree of PAH metabolism by non-liver tissues. Whether this has any bearing on in vivo transformation of HMEC is still unknwn. Of possible note is the fact that the breast consists largely of adipose tissue, in which the lipid soluble PAH can concentrate. Comparisons of BaP metabolite products from 22 different specimen donors showed around a 5-fold range in values. Thus, this is a situation where individual variability needs to be considered. Additionally, we found that culture conditions can significantly influence the metabolites formed. The pattern

of metabolites could vary with medium used (MM vs. MCDB 170), passage level (pre- or post-selection MCDB 170 cells) and use of sub-optimal culture conditions (confluent or overly acidic cell cultures).

We also showed that the damage resulting from BaP metabolism may be due to oxidative damage as well as bulky adduct formation. We found that the lethal effect of BaP correlated with the extent of thymine glycol formation (a measure of oxidative damage) rather than bulky adduct formation, and could be reduced by agents which protect against oxidative damage, such as superoxide dismutase.

V.B. Calmodulin-Like Protein (references, Yaswen et al., 1990; Yaswen et al., 1992; Edman et al., 1995)
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One approach we took to characterize the differences between our normal and transformed HMEC cultures was to use subtractive hybridization to identify genes expressed in the normal HMEC and downregulated in the immortal and malignantly transformed cells. Subtractive hybridization was performed between the normal 184 cell cDNA and both 184B5 and B5KTu cell mRNA. In addition to identifying fibronectin, keratin 5, and vimentin, a 350 base pair cDNA fragment was isolated which initially showed no similarity to any sequence reported in GenBank. This cDNA hybridized specifically to a 1.4 kb mRNA, designated NB-1, which was expressed in normal HMEC, but was downregulated (184B5) or undetectable (184A1) in the transformed cell lines. Sequence analysis of a full length NB-1 clone revealed a 447 bp open reading frame with extensive similarity (70%, 71%, and 80%) at the nucleic acid level to the three known human genes coding for the ubiquitous calcium binding protein, calmodulin. The similarity between the translated amino acid sequence of NB-1 and human calmodulin was 85% over the length of the entire protein.

Using Northern and PCR analysis, NB-1 mRNA has been thus far found only in normal epithelial cells and tissues from human breast, prostate, cervix, and skin. It has not been found in normal epithelial cells other than those from stratified or pseudo-stratified tissues. It was not detectable in non-epithelial cells and tissues, nor in any of the mammary epithelial tumor cell lines which we have examined. Human breast cells obtained from lactational fluids were also negative for NB-1 expression by PCR analysis.

Expression of NB-1 mRNA is not significantly decreased when cells are growth arrested by exposure to anti-EGF receptor antibodies or in senescing cells where proliferation is minimal. It is increased in cells growth arrested by TGFb and reduced when HMEC are grown on reconstituted extracellular matrix material.

Using antisera which displayed a strong preference for NB-1 protein over calmodulin., the level of endogenous NB-1 protein in 184 HMEC was approximately 100-200 ng / 10^-6 cells, a level similar to the estimated level of calmodulin in other cultured cell lines. The relative abundance of the 16kD NB-1 protein (named calmodulin like protein, or CLP) reflected relative NB-1 mRNA levels in various cell types. In contrast, levels of calmodulin protein were nearly constant in the same cell extracts.

Using indirect immunoflourescence, CLP was shown to be present diffusely throughout the cytoplasm and, to varying degrees, in the nuclei of 184 interphase cells. During mitosis, CLP was particularly bright in regions around mitotic spindles. In 184B5, CLP expression was heterogeneous both among different cells and within individual cells; no significant CLP immunofluorescence was observed in 184A1. In surgical specimens from histologically normal breast tissues, CLP staining was strong in the majority of basal cells from small ducts. Luminal cells in the small ducts showed some staining, although not as intense. In larger ducts, staining was mainly confined to the basal cells and was generally less intense than in the small ducts. In all cases, distribution of the protein appeared uniformly intracellular. No staining was evident in basement membrane or stromal areas. In contrast to normal breast tissue, sections from six infiltrating ductal breast carcinomas were consistently negative for CLP expression. Serial sections of the normal and tumor tissues all showed abundant calmodulin expression.

CLP distribution was also been examined in other stratified and pseudo-stratified epithelial tissue sections. In normal prostate, nearly all the epithelial cells were stained to a similar degree. In normal cervix and skin, no staining was observed in the basal cell layer. In the cervix, suprabasal cells were intensely CLP positive, with the degree of staining diminishing in the more distant upper layers. In the skin, the intensity of staining increased from the suprabasal layer until the stratum corneum, which itself was not stained. Thus, in the four different tissues examined, CLP showed distinct patterns of expression. These results suggest that the role of CLP may be defined by the differentiated state of the cells where it is expressed.

An unusual feature of CLP genomic DNA is the absence of introns, whereas all vertebrate calmodulin genes studied to date contain five similarly placed introns. It is possible that CLP may be a rare example of an expressed retroposon.

External calcium concentration has been shown to affect the proliferative potential and differentiated states of some cultured epithelial cells, including keratinocytes and mammary epithelial cells. In normal keratinocytes, increasing calcium concentrations can lead to cessation of proliferation and expression of markers of terminal differentiation, and loss of response to the calcium induced differentiation signal has been shown to correlate with the early stages of transformation. The downregulation of CLP expression observed after in vitro and in vivo transformation of HMEC may reflect a consequence of, or a requirement of the transformed state. Possibly, a particular state of differentiation is required for transformation to occur, or the transformed state may be incompatible with high expression of CLP.

VI. Information on HMEC Computer Records, Mailing Sheets, and Distribution Index

In order to accurately record both the many varieties of HMEC being used in my lab, and the cell cultures distributed to others, it became acutely necessary to develop appropriate record keeping practices. These have been threefold: (1) A complex relational database for recording frozen cell culture inventories and information; (2) A simple database for recording cell cultures distributed to other laboratories and a newsletter and other informational material on cell usage to be distributed along with the cells; (3) Standardized record keeping formats for my lab. More details are presented below.

VI. A. Cell Inventory Database

When I first starting collecting and freezing cell material in the days before personal computers, records were maintained on index cards on rolodexes. This was obviously not an ideal format, particularly as the number and complexity of cell types increased. We acquired a PC in 1985 and I worked with a programmer to develop a suitable inventory database using DBase. Since I am not familiar with computer programming, DBase on a PC was not an optimal situation for me. As soon as the Macintosh II and the 4th Dimension database were available in 1987, the existing program and records were transferred and refined. I worked closely with a computer programmer to design the 4th Dimension program, which is flexible enough to allow me to make adjustments in layout, data organization, and ways to select and present data. More recently, we have been able to have a computer programmer make major updates and improvements to this program.

The database consists of two main related files. One file (Inventory) records the complete identity of each frozen cell batch (identified by Specimen ID, Cell, Tissue, Passage #, and, where relevant, also Type, Subtype, FreezeDown Symbol and Selection), the number of ampoules of that batch which were made and which remain, the location of that batch of ampoules in the freezer, and a space for comments. Whenever cells are frozen, a test ampoule is included. When the test (or the first ampoule of that batch) is removed it is scored for viability, health, and the number of days it takes to reach confluence. This information is then entered into the Inventory file (unfortunately, not all batches have been tested, so sometimes I end up sending cells whose viablilty has not been ascertained). All freeze downs are also tested for mycoplasma contamination by Hoechst staining, and the results of this testing are entered into the Inventory file. This program allows for easy selection and sorting on any field.

The second file (Location) records the location of each individual ampoule. When an ampoule is removed, it records and files the information on date, purpose for removal, and who removed them. It also updates the Inventory file records to indicate the reduced number of ampoules remaining.

In both files, all ampoules are identified by a 5 digit code, the **FreezeDownNumber (FDN)** you see on the Mailing sheets I send. The purpose of this code is to encapsulate all of the information above into 5 digits that can be easily written on an ampoule and stored in a computer. **IT DOES NOT BY ITSELF IDENTIFY THE CELLS. PLEASE, DO NOT REFER TO THE CELLS BY THIS CODE!** I have no idea what cells you're talking about without checking the database. These code numbers have even crept into publications - a truly confusing situation. The cells should be referred to by their Specimen Identification, Type, and Subtype (sent to your on the mailing sheets, see below).

VI. B. Cell Distribution Database

I use simple databases I created in Panorama II to keep track of cells sent to other investigators, (Recipients

file) and to keep addressses and information for the Newsletter (Newsletter file). The Recipients file generates the mailing sheets that go along with the distributed HMEC. These databases allow for easy selection on all fields. Figure 10 gives an example of these mailing sheets and explanations of the categories.

Figure 10. Example of a HMEC Mailing sheet.

I started the Newsletters as the number of collaborations and those requesting cells increased. Basically, I wanted to ensure that a certain level of information about the cells was given to each investigator. Additionally, I thought it might be helpful for all those using the same cell system to be aware of what others labs were doing. Output of the Newsletters has been rather sporadic. One of the goals of this EMail bulletin Board is to replace the Newsletters. Thus, this review is intended to provide the basic information (and more) that was in the newsletters and in reviews that I would send to investigators requesting cells. Additionally, methods for use of the cells (see **Procedures**) and the list of other investigators and their research subjects is available (see **HMEC Investigators List**). It is my hope that others with resources and methods useful for studies with HMEC will also post their information. We also are working to make posting of anyone's information on publication/research/comments/queries easy and interactive.

VI. C. Cell Distribution

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Given the large numbers and types of cells available, I prefer to talk individually with each person desiring cell cultures to determine what is most appropriate for their needs. If you want cells, you will be asked to send a brief (1 page) letter describing your planned experiements, and indiciating that (1) you will keep me informed of results or major changes in planned experiments; (2) you will not give the cells to others without my permission. Let me know in you want to be on the Investigators List, and if so, include all the information as you would want it listed (including EMail address). There are also legal forms from the University of California for you and your institution to sign and return. I will want from you a FedEx number or equivalent to charge the costs of shipping the cells. We prefer to send out frozen cell cultures; these are placed in dry ice and send overnight. We can also send live cells at room temperature if absolutely necessary, but we seriously discourage this alternative. A mailing sheet will be sent with the cells (see above). We have not tested every freezedown (particularly the more obsure) so occasionally we will be sending cells that have not been tested for viabilty. I will note this on the mailing sheet. Otherwise, the cells that are being sent are known to be capable of good growth under the appropriate culture conditions.

General Cell Culture Reminders:

Taking care of normal human epithelial cells in culture bears some resemblance to taking care of children - the cells may behave by their own logic and timing, which may not coincide with that of the care provider. To optimize the accuracy and consistancy of experimental results, the cell's needs have to come first. Some of the important things to remember for these HMEC: (1) pH must be carefully controled. The color of the pH indicator should be around salmon-orange. Yellow indicates too acid conditions; in my experience cells left at such acidity become irreversibly sick. The HMEC quickly acidify the culture medium, particularly when near confluent. We change the medium every 2 days (3 on weekends) and refeed a culture 24 hrs before subculture or experimental usage. Your results may differ if you use cells that are acidic or haven't been fed in a while. (2) The cells do not stay healthly once they become confluent. They should be subcultured when subconfluent or just confluent. We use subconfluent cultures for most biochemical and molecular studies. Your results may differ if you use confluent (non-proliferating) cultures. (3) Some cell biology changes as a function of age in culture. It is best to repeat experiments using cells at around the same passage level, with the same life expectancy. This is also a good practice for the cell lines, which may change over extended periods of time in culture. Your results may differ if you use cells at very different passage levels. (4) It's helpful to look at the cells frequently, to become familiar with how they appear under different circumstances. An enormous amount of useful information can be gleaned simply by careful visual observation. If something doesn't look right, it probably isn't, and should be investigated immediately.

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Procedures for Culture of Human Mammary Epithelial Cells

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I. Cells grown in serum free medium.

(references: Hammond et al. 1984, Stampfer 1985)

A. Making media and stocks.

We obtain our media, originally designated MCDB 170, from CLONETICS (1-800-85CLONE;); other sources of MCDB 170 are available.

The media from Clonetics comes in various forms with different names. Their version of complete MCDB 170 with most of the serum-free supplements is called MEGM, Mammary Epithelial Growth Media. **NOTE:** MEGM does not contain all the serum-free supplements that we use, i.e., it does not contain transferrin and isoproterenol. These need to be added. Also, the BPE is supplied separately. Clonetics also provides a medium lacking most of the serum-free supplements, which is called MEBM, Mammary Epithelial Basal Medium. NOTE: this medium does contain the ethanolamine and phosphoethanolamine - do not add these. Additionally, the media can use either a sodium-bicarbonate buffer base, or a HEPES buffer base, called MEBM-SBF. **We highly recommend you use the HEPES based (sodium-bicarbonate free) media if at all feasible.** Clonetics also provides a phenol-red free medium, called MEBM-PRF. The serum-free supplements that need to be added to each 500 ml bottle (if not already present) are as follows:

Supplement	Amount/Bottle	Final Concentration
EGF (20,000X stock, 100ug/ml)	25 ul	5 ng/ml
Hydrocortisone (2000X stock, 1 mg/ml)	0.25 ml	0.5 ug/ml
Insulin (200X stock, 1 mg/ml)	2.5 ml	5 ug/ml
BPE (200X stock, 1400 mg/ml)	2.5 ml	70 ug/ml
Transferrin (2000X stock, 10 mg/ml)	0.25 ml	5 ug/ml
Isoproterenol (500X stock, 5x10^-3 M)	1.0 ml	10^-5 M
Ethanolamine (1000X stock, 10^-1 M)	0.5 ml	10^-4
O-Phosphothanolamine (1000X stock, 10^-1M)	0.5 ml	10^-4M

Maintenance of the appropriate pH is critically important. We have kept cells grown in the original formulation of MCDB 170 (HEPES based) in incubators with low (0.1-2%) CO2 settings. At these low settings there may be considerable variation among incubators. Ultimately, proper pH is best determined by color indicator: media should be salmon orange. **Yellow is too acid,** red is too basic. Clonetics media with bicarbonate is designed to make it easier for people to use the cells at the more typical setting of 5% CO2. However, the cells do not grow as well with the bicarbonate base, do not maintain their pH levels as well inside or outside the incubator, and can be less efficient for certain procedures (e.g., calcium phosphate mediated transfection). I **strongly** recommend that you obtain the HEPES buffered media if you can designate an incubator to be used at the low CO2 setting. Cells growing serum free are more sensitive to toxins in the environment. Be sure you have not placed in or used for cleaning your incubator any potentially toxic compounds. Washing the incubator with distilled water and then leaving the door open for a day can alleviate some problems.

MCDB 170 is also available from the UCSF Cell Culture facility (415 476-1450). While this media is cheaper, it requires more effort to put together (check with me for instructions) and there is significantly less quality control (e.g., the media is not tested on the mammary cells). Other commercial sources provide powdered MCDB 170.

Serum-free Supplement Stocks for MCDB 170

BPE (Bovine Pituitary Extract) 200x stock, 1400 mg/ml

We obtain our BPE from Susan Hammond, 510 522-3625, P.O.Box 147, Alameda, CA 94501. This is the cheapest source we know but requires some processing. BPE is also available ready to use from Clonetics and other commercial sources.

1. Take bottles (100 ml) "raw" BPE out of freezer and put in 37 C water bath to warm up.
2. Transfer BPE to several Oak Ridge tubes and balance pairs.
3. Centrifuge using adaptors in JA-20 rotor at 15K rpm for 30 min., 4 C . Make sure that the "O" rings on the rotor are in place.
4. Pour the supernatant into clean Oak Ridge tubes. Rebalance. Centrifuge again at 15K for 10 min. more. If supernatant doesn't appear to be clear, centrifuge 10 min. more, same conditions.
5. Transfer supernatant from all tubes into flask. Discard the pellet at the bottom of the tubes. Filter the supernatant through 0.8 µm, 250 ml Nalgene filters, about 30 ml at a time; then filter through 0.2 µm, 115 ml Nalgene filters. Several will be needed because they get clogged.
6. Label sterile 50-ml and snap-top polypropylene tubes with "BPE, 200X, month/year, vial letter, i.e. A, B, C, etc." and include an expiration date.
7. Aliquot 26 ml and 2.8 ml portions into sterile polypropylene tubes.
8. Do sterility check on each vial, using 10 µl from each aliquot into 1.5 ml sterile media, check test every day for 4 days.
9. Store in labeled rack in -20 °C

EGF (Epidermal Growth Factor) 20,000x stock, 100 µg/ml

We obtain Human EGF from Upstate Biotechnology. Many other commercial sources are available

1. Retrieve 100 µg vial of hEGF from refrigerator. Make sure that the vial is unopened and actually says 100 µg.
2. Make up to 0.1 mg/ml by adding 1.0 ml sterile distilled water to vial. Mix gently, but well. If necessary, vary the concentration according to the weight in the vial.
3. Aliquot 0.26 ml portions into sterile ampoules labeled "hEGF, 20,000X, Month/Year, ampoule letter, i.e. A,B,C etc."
4. Check sterility of each ampoule by adding 3 µl from each ampoule to 1.5 ml media in a 35 mm dish and incubate 3 or 4 days. Check every day for contamination.
5. Store in -20 °C freezer for up to 3 months

Transferrin Human 2000x stock, 10 mg/ml We obtain from Sigma CAT# T-2252 Siderophilin

1. Dissolve 1000 mg of transferrin into 100 ml distilled water. This gives a stock concentration of 10 mg/ml.
2. Filter for sterility through 0.2 µm filter.
3. Aliquot 2.6 ml and 0.30 ml portions into sterile polypropylene tubes or snap-top tubes labelled "Transferrin, 2000x, month/year, vial letter, i.e., A,B,C, etc."
4. Do sterility check by placing 10 µl from each vial into corresponding labeled 35 mm dish with 1.5 ml media, check every day for 4 days.
5. Store at -20 °C freezer.

Isoproterenol 500x stock, 5x10-3M We obtain powder from Sigma CAT #: I-5627 (+ Isoproternol: hydrochloride crystalline) which is stored at room temperature.

1. Make up in hood. Do not inhale dust. Measure in hood or in covered balance. To make up 40 ml, use 50 mg IP in 40 ml 95% EtOH.
2. Store stock in freezer: Make up fresh monthly.

Insulin 200x stock, 1 mg/ml We obtain from Sigma CAT # I-5500

1. Dissolve 1g of powder in 200 ml of 0.005 N HCl (1 ml 1 N HCl with 199 ml of distilled water) by stirring on a magnetic stirrer.
2. When the solution is clear*, add 800 ml of distilled water, to make the final concentration of insulin 1 mg/ml.
3. Sterilize by filtering through a 0.2 µm filter.
4. Label approximately 30 sterile snap-top tubes and enough 50-ml sterile polypropylene tubes with "Insulin, 200X, month/year". Aliquot 2.8 ml and 26 ml portions into sterile polypropylene tubes.
5. Store at -20 °C.

* If the solution is not clear after a reasonable amount of stirring, add a few more drops of 1 N HCl. (The total [HCl] should not exceed 0.005 N HCl/ liter of solution.) When the solution clears, then bring up to 1 liter with distilled water.

Hydrocortisone 2000x stock, 1 mg/ml We obtain from Sigma CAT #H-4001

1. Add 50 mg Hydrocortisone to 50 ml 95% ethanol, mix well.
2. Store at -20 °C in a sterile 100 ml glass bottle, no sterility test necessary.

Ethanolamine (2-Aminoethanol) 1000x stock, 10- 1M We obtain from Sigma: CAT# A-5629

1 Dissolve 341.6 mg ethanolamine (approx 0.15 ml) in 56 ml MCDB 170 base to give a final stock concentration of 6.1mg/ml or 0.1M.
2. Filter through 0.2 µm filters for sterility.
3. Label sterile polypropylene tubes "Ethanoloamine, 1000X, mo./yr, vial letter" and aliquot 5 ml and 0.6 ml portions.
4. Do sterility test by aliquoting 5µl from each tube into corresponding labelled 35mm dish with 1.5 ml media. Check every day for four days.
5. Store at -20 °C.

O-Phosphoethanolamine 1000x stock, 10- 1M We obtain from Sigma CAT# P-0503 .

1. Dissolve 789.5 mg in 56 ml of MCDB 170 base to give a stock concentration of 14.1 mg/ml or 0.1M.
2. Filter through 0.2µ filter for sterility.
3. Label sterile polypropylene tubes "phospoethanoloamine, 1000X, mo./yr, vial letter" and aliquot 5 ml and 0.6 ml portions.
4. Do sterility test by aliquoting 10µl from each tube into corresponding labelled 35mm dish with 1.5 ml media. Check every day for four days, repeat if necessary.
5. Store at -20 °C.

NOTE: Also spelled 'O-Phosphoroethanolamine' and 'O-Phosphorylethanolamine

B. Growing and Subculturing Cells in Serum-free Medium

1. Feeding and Plating Volumes and Densities (for routine culture):

Dish/Flask	Amount Media (ml)	Number of Cells for Seeding
T-75	12-15	3-5 X 10^-5
100mm	10-12	3-4 X 10^-5
60mm; T-25	3.5-5	1.0-1.5 X10^-5
35mm	1.5	0.5 X 10^-5
24 Well Plate	1.0/well	2 X 10^-4/well

Cells plated at these densities will increase 6-10x in number before confluence. We routinely grow cells in dishes, **not** flasks, because of the better gas exchange. We only use flasks if cells need to be transported.

2. Seeding Cells from a Frozen Ampoule (Non Organoids):

1) Label and add medium to all dishes
2) Put 2 scoops of liquid nitrogen into styrofoam box containing ampoule rack and cover with lid.
3) Remove cells from freezer and immediately place in box, cover with lid.
4) Thaw one ampoule at a time in 37 °C water bath, do not immerse top into water. As soon as it is thawed, wipe down with 70% ethanol in the hood.
5) Open amp and mix well with added media for a good distribution into the dishes, i.e., if seeding 5x10^-5 cells into 3-60's, assume that there is 0.5 ml in the amp already and add 1.3 ml media, mix and distribute 0.6 ml to each 60 mm dish. It's most important to mix cells well and distribute cells evenly.
6) Distribute cells evenly by gently pushing dish back and forth 8-10 times in each direction. Check distribution under the microscope. Place in incubator.

We do not pellet cells thawed from the freezer and we do not recommend that you do either. Cells are frozen in 10% glycerol plus 15% FCS (no DMSO).

3. Subculture of Cells

It is important to subculture the cells when they are subconfluent or just confluent, as they lose viability when kept in confluent cultures. We do most of our experiments, or harvest cells for RNA and DNA, when they are subconfluent and still actively proliferating. When the cells are growing as they should, they are subcultured around once a week at 1/6 to 1/10 split ratios. If your cells are not growing this fast, *something is wrong.* Call!

We do not subculture all cells of one specimen at the same time. e.g., if there are 3 60s, we subculture 1 one day, and wait 48 hrs before discarding the extra dishes, or, wait 24 hrs to freeze the extra dishes. If you must split all the cells the same day, I recommend doing it in two separate, (non-cross contaminatable) batches.

NOTE ON TRYPSIN: Always label trypsin with the date thawed; do not use trypsin more than one week old. Do not forget to take trypsin out of 37 °C water bath immediately when thawed. Better yet, give it the time to thaw at room temperature. Stor e at 4 °C.

The most common error I have observed is not following the instructions for subculture. DO NOT IMPROVISE! Follow these instructions. These cells are very tightly attached to the substratum

and need strong measures to be removed.

Need:

- Sterile pipettes
- Plugged/unplugged Pasteur pipettes
- Sterile STV (Saline base; Trypsin 0.05%; Versene (EDTA) 0.02%
- Sterile PBS
- Sterile 15 or 50 ml centrifuge tube
- Hemocytometer (clean)
- Dishes or flasks

1) Look at cells under microscope. Make sure they are not contaminated and look appropriate.
2) Aspirate media from parent dish. **Wash once with STV (not something else):**

- 100 mm dish, T-75 flask: use about 3 ml
- 60 mm T-25: 2 ml
- 35 mm: 1 ml

3) Add just enough STV to barely cover the cells (important to minimize amount of STV), in general:

- 100 mm, T-75: 1-1.2 ml
- 60 mm, T-25: <= 0.4 ml
- 35 mm: <= 0.2 ml

4) Incubate cells in **37 °C.** incubator for 2-5 minutes. This time is not fixed, as cells vary. Do not leave at room temperature. The cells will not come off at room temperature.
5) Take cells out of incubator and check under microscope to see if they have all "rounded up". Don't leave the STV on longer than necessary to remove most of the cells - trypsin chews up cells. Tap dish lightly against hand or desk if necessary to kn ock cells loose. Continue incubation at **37 °C.** if cells are still attached.
6) When most of the cells have loosened add PBS to culture vessel, preferably using a plugged Pasteur pipette with a hand pipetter, or otherwise regular pipettes. Do not wait for all the cells to come off, particularly if only small patches remain, or in the case of 184B5, which tends to hold on tightly. Repipette the PBS in the flask/dish to break up clumps, and then transfer this to 15 or 50 ml tubes (use the larger volume tube for 100mm dishes, T-75. or more than 2-60mm dishes). Wash the flask/dish with about the same amount of PBS one or two times and transfer to tube. The final volume in the tube should be about:

- 100 mm, T-75: 6-12 ml
- 60 mm, T-25: 3-6 ml
- 35 mm: 1-3 ml

depending upon the cell density of the culture
7) Repipette to mix and to break up any cell clumps to facilitate counting single cells. Take a small amount in the tip of a plugged Pasteur pipette and drop onto both sides of a haemocytometer. *Note the volume in the centrifuge tube.*
8) Check under microscope that both chambers of haemocytometer have approximately the same number of cells (low power). The ideal # of cells to count is about 100 cells/5 squares/chamber.
9) Bring up volume of 15 ml or 50 ml test tube to maximal with PBS to dilute STV.
10) Centrifuge the cells in a table top centrifuge at 800-1000 rpm for approximately 5 minutes.
11) While cells are centrifuging, count cells in haemocytometer (10X power). We count 5 squares per chamber x 2 chambers. Record # of cells counted/chamber, and determine total cell count (using the noted volume in the centrifuge tube). Based on what's to be done with cells, calculate dilutions to be performed when cells are resuspended. Label dishes/flasks to be seeded, and add necessary amount of medium.
12) Carefully aspirate the PBS/STV away from the cell pellet.
13) Bring the cells up to the desired cell density with:

- a) Media if you are subculturing the cells. The density of dilution depends upon seeding protocol. We generally dilute to either about 10^-5 cells/ml, or we add back the same volume as was present for cell counts to simplify calculations. Pipette a few times with 1 ml pipette to get rid of cell clumps. Seed dishes and gently swirl medium to insure even distribution of cells in the culture vessel. Place in

incubator.

- b) Freeze media if you are freezing the cells (see below).

4. Freezing Cells for Liquid Nitrogen Storage

We always freeze our cells at the density of 10^-6 cells/ml freeze medium. Cells are generally frozen in 0.5 or 1.0 ml amounts (5 X 10^5 or 10^6 cells), depending on number of cells to be frozen and what cells are to be used for, but other quantities are O K if necessary.

We also make a test ampoules with every freezedown. The test contains 1.7 X 10^5 cells/0.17 ml freeze media, or, 1/3 the usual amount of 0.5 ml. They are seeded into 3 35 mm dishes one week after storage of freeze-down in liquid nitrogen to determine the viability and health of the cells in that freezedown.

We feed cells the day before they are to be frozen. If there are multiple dishes-some of which are to be subcultured, we split those one day, and the next day check to see if they are OK (not contaminated). If so, then we go ahead and freeze the remai ning dishes.

1) Follow subculture procedure to # 12
2) Cells growing in MCDB 170 are frozen in Glycerol I freeze media. This must be kept on ice during use.

Glycerol I : stored at -20 °C; kept at 4 °C. after thawing. To make 200 ml:

Glycerol	20 ml	10%
FCS	30 ml	15%
MCDB 170 base	150 ml	75%

Mix in T-75 flask. Filter (0.2 µm) for sterility. Do sterility check.

3) Add the appropriate amount of Gly I to cell pellet to have 10^-6/cells/ml. Keep on ice.
4) Label ampoules for freezing. Add appropriate amount of cells to each ampoule. Keep ampoules on ice.
5) We use a very low-tech freezing method. It's probably not the best, but it works. Ampoules are wrapped in Kim Wipes and placed in a styrofoam cup lined with crushed Kim Wipes to keep them from freezing too quickly, and then covered with foil.
6) The cup with ampoules is placed in a -70 °C. freezer for 24 hours.
7) Ampoules are transferred to liquid N2 *within one week.*

II. Cells grown in serum-containing MM medium.

(references: Stampfer et al., 1980; Stampfer, 1982; Stampfer 1985)

A. Making media and stocks

The composition of MM Medium as originally formulated is as follows:

Component	Amount/500 ml	Final Concentration
1:1 mixture of DME/F-12	300 ml	
Hs767Bl and/or fHs74Int (**)	100	20%
Hs578Bst (**)	50	10%
FCS	2.5 ml	0.5%
Insulin (100x stock; 1 mg/ml)	5 ml	10 µg/ml
EGF (20,000x stock; 10 µg/ml)	25 µl	5 ng/ml
Hydrocortisone (10,000x stock; 1 mg/ml)	50 µl	0.1 µg/ml
Cholera toxin (10,000x stock; 10 µg/ml)	50 µl	1 ng/ml
T3;triiodothyronine (20,000 stock; 2x10-4M)	25 µl	10^8 M
E2;¤-estradiol (20,000x stock;2x10-5 M)	25 µl	10^9 M
Pen-Strep (100x stock)(optional)	5 ml	

** Conditioned Media: (Cells grown in 1:1 DME/F-12 with 5% FCS + 5 µg/ml insulin)

The above represents MM medium as originally formulated. We have used many variations on this (unfortunately not all given clearly distinct names). Nowadays, we often omit the T3 and E2 from the formulation. We do not have good experimentation to indicate the effect of these components.

MM without any conditioned media (CM) is referred to as MM4. The cells will proliferate without the CM as long as the cholera toxin is present. The CM can effect both the long-term growth of the cells and their state of differentiation. Nowadays we often use only Hs767Bl CM (we have frozen stocks of these cells). Early experiments suggested that the CM from Hs578Bst served mainly to help cell attachment.

The DME/F-12 base is buffered with sodium bicarbonate, generally requiring around 5% CO2 settings for the incubator. However, we have found that actively growing cultures, particularly as they approach confluence, tend to overwhelm the acidic buffering capacity. This can be seen by the phenol red indicator - culture medium becomes yellow. Therefore, we often kept a separate incubator set at lower CO2 concentration (around 3%) to which we removed cells as they became more confluent. Frequent refeeding could also alleviate the acidity. The cells will lose viability if left acid for too long.

Stocks for MM

(not already listed for MCDB 170)

Cholera Toxin 10,000x stock (10 µg/ml) We obtain from Sigma CAT# C3012

1.Add 0.5 ml sterile distilled water to 0.5 mg of cholera toxin in bottle to yield 1 mg/ml.
2. Dilute this 1:100 (0.1 ml/10 ml) in sterile distilled water to yield 10 µg/ml. Leave in concentrated form because it loses activity less quickly.
3. Aliquot into 1 ml ampoules.
4. Store in refrigerator for up to 3 months; **do not freeze.**

T3; 3,3', 5-tri-iodo-L-thyronine 20,000x stock (2x^10-4M) We obtain from Sigma CAT# T-2877.

1. Dissolve 1.302 mg in 10 ml distilled water. Add 1 drop weak NaOH (0.1 M to 0.5 M). Filter (0.2 µm) sterilize. Refrigerate. Make up fresh monthly.

E2; beta-estradiol 20,000x stock (2x10^5 M) We obtain from Sigma CAT# E-8875

1. Dissolve 5.44 mg in 10ml absolute EtOH to make 2 x 10-3M. (MW = 272.4).
2. Dilute 100 µl of this into 10 ml for a 2x 10-5 M stock. Store in freezer at -20 °C.

B. Growing and Subculturing Cells in MM

1. Feeding and Plating Volumes and Densities:

Feeding and plating densities are similar to cells in MCDB 170 with one major exception. MM does not support clonal growth of HMEC. Seeding at densities lower than those indicated will lead to reduced cell viability.

2. Seeding Cells from a Frozen Ampoule

The procedure is the same as for cells grown in MCDB 170 except that the cells grown in MM have been frozen in 1:1 DME/F-12 with 15% FCS and 10% DMSO.

3. Subculture of Cells

See general notes for cells grown in MCDB 170. The main difference for cells grown in MM is that the presence of serum in the medium eliminates the need to pellet the cells to remove all traces of the trypsin.

1) Look at cells under microscope. Make sure they are not contaminated and look appropriate.
2) Aspirate media from parent dish. **Wash once with STV (not something else):**

- 100 mm dish, T-75 flask: use about 3 ml
- 60 mm T-25: 2 ml
- 35 mm: 1 ml

3) Add enough STV to barely cover the cells, in general:

- 100 mm, T-75: 1-1.2 ml
- 60 mm, T-25: <= 0.4 ml
- 35 mm: <= 0.2 ml

4) Incubate cells in 37C. incubator for 2-5 minutes. This time is not fixed, as cells vary. Do not leave at room temperature. The cells will not come off at room temperature.
5) Take cells out of incubator and check under microscope to see if they have all "rounded up". Don't leave the STV on longer than necessary to remove most of the cells - trypsin chews up cells. Tap dish lightly against hand or desk if necessary to kn ock cells loose. Continue incubation at 37C. if cells are still attached.
6) When most of the cells have loosened add MM to culture vessel, preferably using a plugged Pasteur pipette with a hand pipetter, or otherwise a regular pipette. Do not wait for all the cells to come off, particularly if only small patches remain. Repipette the MM in the flask/dish to break up clumps, and then transfer this to 15 or 50 ml tubes. Wash the flask/dish with about the same amount of MM one or two times and transfer to tube. The final volume in the tube to optimize cell counts should be about:

- 100 mm, T-75: 6-12 ml
- 60 mm, T-25: 3-6 ml
- 35 mm: 1-3 ml

depending upon the cell density of the culture
7) Repipette to mix and to break up any cell clumps to facilitate counting single cells. Take a small amount in the tip of a plugged Pasteur pipette and drop onto both sides of a haemocytometer. Note the volume in the centrifuge tube.
8) Count cells in haemocytometer (10X power). We count 5 squares per chamber x 2 chambers. Record # of cells counted/chamber, and determine total cell count (using the noted volume in the centrifuge tube). Based on what's to be done with cells, calculate dilutions to be performed. Label dishes/flasks to be seeded, and add necessary amount of medium.
9) Seed dishes and gently swirl medium to insure even distribution of cells in the culture vessel. Place in incubator.

4. Freezing Cells for Liquid Nitrogen Storage

See notes for cells grown in MCDB 170. Follow the same procedures (including the subculture procedure for

cells grown in MCDB 170). The only difference is that we have used a different cell preservative medium (CPMI) for cells grown in MM. Additionally, it is not necessary to use just PBS to stop the trypsinization. MM, or buffer/medium containing FCS can be used.

CPMI : stored at -20oC; kept at 4oC after thawing. To make 200 ml:

DMSO	20 ml	10%
FCS	30 ml	15%
DME/F-12 base	150 ml	75%

Mix in T-75 flask.

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APPENDIX C

Dear Colleague,

I am pleased to report that I am finally getting my Home Page on Human Mammary Epithelial Cells (HMEC) on-line. You can find this Page on the world wide web, the address is http://www.lbl.gov/~mrgs.

At the moment, what I have posted is just information from my laboratory. This consists of a listing of the HMEC that I have available for distribution, an extensive review providing all (and more) of the basic information on the derivation and characterization of these cells, and protocols for growth and use of these cells. If you are currently using, or plan on using these HMEC, you can download this information (thus saving me the need to continually mail this to others). There is also a list of investigators involved in HMEC research. This file still needs some work. If you want some corrections in your listing (or want to be listed) please let me know.

The next steps will be to post more information and protocols from other labs using HMEC. I have some material that was sent me last spring and summer (sorry it has taken so long). I now welcome any further information you would like to see posted (e.g., abstracts from papers or meetings, reports of research progress, queries to others, specific protocols for uses of HMEC). You can send this to my Email address, and we will format for the WWW. Beyond that, we are hoping to create an interactive page, where queries and responses can be made directly.

Let me know if you have any further ideas or suggestions.

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